UNIVERSIDAD COMPLUTENSE DE MADRID
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ESTUDIO HODOLÓGICO E INMUNOHISTOQUÍMICO DE LAS PROYECCIONES ASCENDENTES SOMATOSENSORIALES EN EL SISTEMA NERVIOSO CENTRAL DE LOS ANFIBIOS

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A Mónica

A mis padres y hermanos

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CAPÍTULO 1

Las funciones del sistema nervioso de los vertebrados incluyen la percepción de distintos estímulos del medio que les rodea, lo que permite la elaboración de respuestas adecuadas para cada tipo de situación, permitiendo así su relación con su entorno. Los estímulos ambientales son captados por los receptores sensoriales, encargados de transducirlos a un código bioeléctrico de potenciales de acción inteligibles para el sistema nervioso, y transportarlos por medio de los nervios sensitivos o aferentes al sistema nervioso central, el cual, después de procesar y analizar la información en diversos centros específicos, elabora respuestas que son conducidas a través de los nervios eferentes hasta los órganos efectores.

Introducción histórica

El filósofo y naturalista griego, Aristóteles (322 a. de C.), describió los **sentidos** en su obra *Historia de los animales*, como las vías o los canales a través de los cuales los animales perciben información de su entorno. Propuso la existencia de cinco sentidos comunes para la mayoría de los animales, entre los que se encontraba el tacto, actualmente considerado una parte integrante del concepto de somestesia o información somatosensorial.

El hecho de considerar las sensaciones cutáneas como una entidad independiente y específica se remonta a los estudios fisiológicos realizados a principios del siglo XIX, (ver Sinclair, 1981), y a las observaciones anatómicas realizadas por Bell (1811). Posteriormente, Magendie (1822) demostró la implicación de las raíces dorsales de los nervios espinales en la transmisión de la información cutánea. En estudios sucesivos, Müller (1840-42), retomando la doctrina Aristotélica de los cinco sentidos, introdujo el término *tacto* como la agrupación de las diferentes sensaciones cutáneas. Sin embargo, durante la década de 1840 a 1850, de acuerdo con Sinclair (1981), varios autores comenzaron a establecer la *teoría de la especificidad*, al distinguir entre los diversos aspectos de la información cutánea, postulando la existencia de terminales nerviosos específicos para cada sensación. Describieron los criterios de irritabilidad selectiva, es decir, cada tipo de órgano sensorial podría ser excitado por un estímulo en particular, que a su vez sería conducido por un nervio concreto. Las citadas hipótesis fueron corroboradas fisiológicamente (Blix, 1884; Goldscheider, 1884, Donaldson, 1885). Blix (1884) demostró que la estimulación específica de distintos puntos con una localización concreta en la piel, producía sensaciones de presión, calor, frio o dolor; y confirmó la especificidad anteriormente propuesta, mediante la demostración de que cada punto responde a una modalidad concreta de estimulación y no a otras. Se localizaron puntos sensoriales que respondían específicamente a variaciones térmicas, a estímulos de presión o de dolor, y se observó que la densidad de la distribución de cada uno de ellos variaba en función de la región corporal analizada (Blix, 1884, Goldscheider, 1884; von Frey, 1896; Dallenbach, 1927). Completando estas valoraciones, von Frey (1906, 1910) postuló la posibilidad de que cada clase de punto sensitivo, podría estar asociado a un órgano sensorial en particular, que previamente había sido identificado histológicamente (Krause, 1859, Meissner, 1859, Ruffini, 1894), sin embargo, su correlación ha sido corregida en estudios

morfofuncionales posteriores (ver Willis y Coggeshall, 1991)

Como consecuencia de la transmisión codificada de los estímulos en las fibras que inervan los órganos sensoriales Adrian (1946), Sinclair (1955) y Weddell (1955) propusieron la *Teoría de los patrones* que sugiere que no es necesaria la existencia de vías o canales específicos para cada modalidad de estimulación cutánea. Por el contrario, la especificidad de la transmisión de los distintos estímulos, vendría determinada por patrones espaciales y temporales de codificación diferencial de la información, empleando las mismas vías. Dicha teoría, sin embargo, ha sido parcialmente rechazada a la luz de observaciones que ofrecen evidencias de la existencia de fibras morfológica y funcionalmente distintas, por ejemplo: mielínicas o amielínicas; o con diámetros y velocidades de conducción desiguales, encargadas de la transmisión de los diferentes estímulos (ver Willis y Coggeshall, 1991). La especificidad presente en las vías anatómicas del sistema nervioso central, en relación a la naturaleza de la información que procesan (Noordenbos y Wall, 1976), apoya igualmente dicha teroría, al demostrar que la información nociceptiva, térmica, y algunos aspectos de la información tactil, son transmitidos a través del cuadrante anterolateral de la médula espinal, contrariamente a las sensaciones mecánicas que ascienden por sistemas espinales diferentes. Los resultados obtenidos en experimentos realizados en humanos con técnicas de microneurografía, consistentes en la evocación de sensaciones mediante la estimulación de una única fibra sensorial periférica, y la posterior identificación de su campo receptivo y del estímulo necesario para su excitación, apoyan igualmente la teroría de la especificidad de inervación de los diversos órganos sensoriales por distintas fibras aferentes (Willis y Coggeshall, 1991).

Consideraciones generales sobre la información somatosensorial

Existen diversas clasificaciones de la información somática, atendiendo a criterios como la naturaleza del estímulo, su procedencia y la posible evocación de sensaciones.

La sensibilidad somatosensorial se divide en información *somática visceral* e información *somática no visceral*. La primera produce sensaciones conscientes como el **dolor visceral**, la **saciedad** producida por estímulos de distensión, así como la percepción del **grado de vaciamiento** de las vísceras; e información no consciente que participa en la regulación del medio interno. La sensibilidad somática no visceral consiste en la percepción de estímulos básicos considerados como *primarios*, en los que se incluyen los que conducen a sensaciones de **dolor, térmicas** y los estímulos **mecanorreceptivos**, que a su vez se dividen en *exteroceptivos* -si provienen del exterior del organismo (**tacto-presión y temblor-vibración**)-, y en *propioceptivos*, -si se originan en el interior del organismo como los responsables de las sensaciones de **posición** y de **movimiento** (**cinestesia**) de los miembros y de las articulaciones-. Asimismo, la sensibilidad somática no visceral incluye también la percepción de estímulos *secundarios* más complejos, como el sentido espacial y el reconocimiento de las formas, resultado de la combinación e integración de la percepción de

estímulos primarios (Mountcastle, 1973; Loewy y Spyer, 1990; Willis y Coggeshall, 1991).

En términos generales, la información somatosensorial puede clasificarse en *especial* y *general* dependiendo de que ésta proceda de la región cefálica o del tórax y las extremidades respectivamente. La información somática *especial* ingresa en el sistema nervioso central a través de las ramas sensitivas de algunos pares craneales (V, VII, IX, X y XII), a diferencia de la información *general* que lo hace a través de las raíces dorsales de los nervios espinales.

En 1920 Head distinguió entre sensibilidad *epicrítica*, cuando se trata de información inocua tactil o de pequeñas variaciones térmicas, y sensibilidad *protopática* para las aferencias nociceptivas o de sensaciones de temperaturas extremas. Dicha nomenclatura, actualmente en desuso (Willis y Coggeshall, 1991), ha sido empleada para calificar y diferenciar algunas vías centrales somestésicas de manera incorrecta, debido a que en estudios posteriores se ha demostrado que ambos tipos de información están entremezclados a nivel de sistema nervioso central.

Igualmente, Poggio y Mountcastle (1960; 1963) describieron los conceptos *lemniscal* y *no lemniscal* con el fin de clasificar patrones diferenciales de respuesta fisiológica en neuronas de los complejos ventrobasal y posterior talámicos respectivamente. Los autores supusieron que las neuronas del complejo ventrobasal eran activadas por el sistema de la columna dorsal-lemnisco medial, mientras que las del complejo porterior lo eran a través del tracto espinotalámico (no lemniscal). Sin embargo, en trabajos sucesivos (Boivie y Perl, 1975) se ha criticado dicha nomenclatura como consecuencia de que tanto algunas de las neuronas que originan el lemnisco medial, como las que proyectan a través del tracto espinotalámico, comparten características que fueron consideradas por Poggio y Mouncastle (1960) como distintivas de ambos sistemas, y a que las dos poblaciones neuronales inervan los complejos ventrobasal y posterior talámicos (Boivie y Perl, 1975).

El término de *modalidad sensorial* fue introducido por Helmholtz (ver Boring, 1942) como una clase de sensaciones cualitativamente continuas, entre las que únicamente existen diferencias cuantitativas como las que presentan dos sensaciones tactiles con intensidades desiguales. En contraposición, las diferencias presentes entre las sensaciones correspondientes a modalidades sensoriales distintas, por ejemplo el tacto y el oido, son cualitativas. Aunque la información procedente de la piel se consideró inicialmente de forma global (Müller, 1840-42), actualmente se distinguen diversas modalidades sensoriales cutáneas: tacto-presión, temblor-vibración, cosquilleo, calor, frio, dolor, picor; así como las subcutáneas propioceptivas que incluyen el sentido de la posición y la cinestesia (Willis y Coggeshall, 1991).

La transmisión de cada modalidad sensorial en la somestesia depende de la activación y el funcionamiento de una o varias vías anatómicas en el sistema nervioso central. Recientemente, Willis y Coggeshall (1991) han introducido el término de *canal sensorial* que engloba todos los mecanismos

necesarios para la percepción de la información relacionada con una modalidad sensorial, con la posibilidad de incluir diversos tipos de receptores sensoriales, vías anatómicas de transmisión de información y regiones cerebrales encargadas de su procesamiento.

MAMÍFEROS

Receptores periféricos en mamíferos.

Los receptores de las distintas modalidades sensoriales fueron inicialmente descritos por Bell (1811) y Magendie (1822) como las prolongaciones periféricas de las neuronas de los ganglios de las raíces dorsales espinales, cuyas prolongaciones centrales se ramifican en la médula espinal. Los receptores se han clasificado en función de la naturaleza y el umbral del estímulo o los estímulos necesarios para su activación, así como por la morfología de sus terminales en los tejidos periféricos, libres o encapsulados, (Perl, 1992) y por las diferencias en cuanto a su comportamiento electrofisiológico (Koerber y Mendell, 1992). Las células ganglionares llevan a cabo la *recepción* de los estímulos, su *transducción* en señales inteligibles, y la *conducción* de las mismas hasta el sistema nervioso central (Perl, 1922) y se clasifican atendiendo a numerosos parámetros.

Morfológicamente, mediante tinciones de Nissl, se distinguen dos grupos de células ganglionares, con la posibilidad de nuevas clasificaciones, atendiendo a observaciones con ténicas de microscopía electrónica. Ambas poblaciones neuronales se han descrito como células grandes que se tiñen débilmente y células de menor tamaño que se tiñen con mayor intensidad (Andres, 1961; Lieberman, 1976; Duce y Keen, 1977; Lawson, 1979; Rambourg y cols., 1983), debido a su diferente contenido en orgánulos citoplásmicos (Yamadori, 1970; Duce y Keen, 1977). Las células grandes y claras se caracterizan por su alto contenido en neurofilamentos y por dar origen a fibras de tipo A. Las células pequeñas y oscuras, por el contrario, tienen un escaso contenido en neurofilamentos y originan fibras de tipo C (ver Lawson, 1992).

Mediante técnicas inmunocitoquímicas y de hibridación *in situ*, se han identificado una gran variedad de neuronas ganglionares en cuanto a su patrón de expresión de uno o varios péptidos, enzimas, filamentos intermedios, neurohormonas, monoaminas, proteínas ligantes de calcio, receptores y oligosacáridos de superficie. Sin embargo, todavía no existe una correlacción clara entre estas subpoblaciones, definidas por su contenido neuroquímico, y los distintos tipos funcionales de células ganglionares, identificados según el destino de su inervación periférica, o por su patrón de terminación en la sustancia gris espinal (Rustioni y Weimberg, 1989; Lawson, 1992; Hunt y cols., 1992).

La velocidad de conducción del estímulo en las fibras aferentes varia en relacción a su diámetro y a su grado de mielinización. Según estos criterios, existen fibras de tipo A, mielinizadas y de alta velocidad de conducción, entre las que se distinguen tres subgrupos (alfa, beta y delta) ordenadas de mayor a menor según la velocidad de conducción y grado de

mielinización (Perl, 1992). En contraposición, las fibras C tienen menor diámetro, son amielínicas y tienen una menor velocidad de conducción (Gasser, 1950, 1955). Las aferencias musculares, sin embargo, se clasifican en los grupos I, II III y IV ordenadas de mayor a menor diámetro (Chang, 1948).

Generalidades sobre la médula espinal de mamíferos: citoarquitectura y patrón de terminación de las aferencias primarias.

La sustancia gris de la médula espinal de mamíferos ha sido dividida en diez regiones denominadas láminas y numeradas del I al X (Rexed, 1952, 1954) atendiendo a criterios citoarquitectónicos como el tamaño, el agrupamiento y la morfología celular. Sin embargo, dicha subdivisión morfológica no refleja una subdivisión funcional (Fyffe, 1992).

A lo largo de todo el espesor del asta dorsal, que incluye las láminas I-VI de Rexed (1952) se ha descrito en cada segmento espinal, un mapa somatotópico de representación de la superficie corporal, correspondiente a un dermatomo (Sherrington, 1898; Brown y cols., 1992), de forma que cada raíz dorsal inerva una región corporal o dermatomo diferente (Brown y Fuchs, 1975; Brown y cols., 1980; Light y Durkovick, 1984; Wilson y cols., 1986). Dentro de este esquema de organización, los nervios que inervan regiones adyacentes de la piel, tienen proyecciones adyacentes en el asta dorsal (Koerber y Brown, 1980, 1982). Según la *hipótesis de la somatotopía presináptica* (Brown y Finch, 1975), debe existir una correlación entre la extensión de los mapas de representación de cada región cutánea, observados electrofisiológicamente, y la extensión de las arborizaciones, en la médula espinal, de las aferencias primarias procedentes de dicha región corporal. Esta hipótesis parece cumplirse al menos en el caso de las aferencias cutáneas (Brown y cols., 1992), aunque podría ser igualmente válida en el caso de las aferencias procedentes de otros tejidos.

El asta dorsal de la médula espinal se puede dividir en tres regiones diferentes, en cuanto a la naturaleza de sus aferencias, lo que podría reflejar su organización funcional. En general, las aferencias mielínicas, A delta, procedentes de los nociceptores cutáneos terminan en las láminas I, V y en la parte externa de la lámina II. Las aferencias A beta, procedentes de los mecanorreceptores cutáneos, se arborizan en las regiones profundas del asta dorsal, que incluyen las láminas III, IV y la zona interna de la lámina II. Por último, las aferencias musculares de tipo I y II, alcanzan las láminas VI, VII y IX y ocasionalmente, la III y la IV (Brown y cols., 1977, 1978, 1980, 1981; Hamano y cols., 1978; Hongo y cols., 1978; Isizuka y cols., 1979; Matsushita y Tanami, 1983; ver Fyffe, 1992 para una revisión). Las fibras C amielínicas terminan mayoritariamente en la lámina II en el caso de los mecanoreceptores cutáneos, mientras que las fibras C nociceptivas, procedentes de receptores polimodales y de frío, se arborizan principalmente en las láminas I y II y en menor medida en la III y la IV. Por último, las aferencias C de origen visceral terminan separadamente en las láminas I, II, V y X (Sugiura 1986, Sugiura y cols., 1989). La *región superficial del asta dorsal* fue identificada por Rolando (1824) a consecuencia de su aspecto gelatinoso, incluye las láminas I y II de Rexed (1952) y representa un centro de procesamiento inicial de la información nociceptiva

(Cerveró, 1989). En esta región superficial se diferencian la zona marginal y la sustancia gelatinosa correspondientes a las láminas I y II respectivamente (Cerveró e Iggo, 1980). En esta zona fundamentalmente convergen aferencias nociceptivas y fibras amielínicas de tipo C. *El asta dorsal intermedia* formada por las láminas III y IV, recibe mayoritariamente aferencias mielínicas procedentes de mecanoreceptores cutáneos, mientras que las *regiones más profundas del asta dorsal* están inervadas por aferencias musculares (Fyffe, 1992).

Proyecciones espinales ascendentes en mamíferos.

En vertebrados terrestres se ha descrito la existencia de dos sistemas básicos de proyecciones espinales ascendentes (Willis y Coggeshall, 1991). 1) El *Sistema columna dorsal-lemnisco medial*, constituído por axones primarios procedentes de neuronas ganglionares espinales, así como por fibras no primarias que forman el sistema postsináptico de la columna dorsal. Ambos tipos de fibras ascienden en el **funículo dorsal** hasta los núcleos de la columna dorsal, situados en la placa alar del rombencéfalo caudal, que a su vez originan el lemnisco medial que asciende contralateralmente para alcanzar diversas dianas rombencefálicas, mesencefálicas y talámicas. 2) *El sistema ventrolateral*, formado por fibras espinales no primarias que ascienden a través del **cuadrante ventrolateral (funículos ventral y ventrolateral)**, hasta la formación reticular, el cerebelo (tracto espinocerebeloso ventral, ver Ito (1984)), el mesencéfalo (Yezierski, 1988), el hipotálamo (Burstein y cols, 1990; Dado y cols., 1994) y el tálamo (Willis y Coggeshall, 1991).

Además, en mamíferos se ha descrito un tercer sistema denominado *espino-cervico-talámico* compuesto por fibras no primarias espinales que ascienden a través del **funículo dorsolateral**, formando el tracto espinocervical que termina en el núcleo cervical lateral. Dicho núcleo origina proyecciones contralaterales que incluyen los tractos cervicomesencefálico y cervicotalámico, que ascienden a través del lemnisco medial hasta el mesencéfalo y el tálamo respectivamente (Willis y Coggeshall, 1991). Asimismo, en el funículo dorsolateral ascienden otras proyecciones como los tractos espinocerebeloso dorsal (Ito, 1984), espinohipotalámico y espinotalámico, y el sistema espino-rombencefálico-talámico (Willis y Coggeshall, 1991).

Los tres sistemas ascendentes están sujetos a un control descendente, a través de proyecciones que se originan en distintas regiones corticales y talámicas, en el núcleo rojo, cerebelo, formación reticular, complejo nuclear del rafe y diversos núcleos rombencefálicos cocleares y vestibulares (ver Willis y Coggeshall, 1991).

Funículo dorsal:

- Sistema Columna Dorsal-Lemnisco Medial.

Dicho sistema está formado por las ramas ascendentes de las aferencias primarias de todos los niveles espinales, organizadas somatotópicamente; es decir, la parte medial del funículo dorsal o fascículo *gracilis* contiene las fibras originadas en niveles espinales caudales a los niveles medios torácicos, que

transmiten información procedente de las extremidades inferiores y de las regiones caudales del torax, y terminan en el núcleo *gracilis*, que ocupa las posiciones más mediales dentro del complejo de los núcleos de la columna dorsal. Únicamente un 20% de las fibras primarias que entran en el fascículo *gracilis*, ascienden hasta el rombencéfalo, mientras que el resto terminan en el asta dorsal, la región intermedia y el asta ventral de la sustancia gris espinal (Glees y Soler, 1951). Las fibras primarias que terminan en la médula espinal, antes de penetrar en la sustancia gris, ascienden diferentes distancias en el funículo dorsal, dependiendo del tipo de receptor periférico y de la naturaleza de la información transmitida (ver Willis y Coggeshall, 1991). Las aferencias procedentes de segmentos torácicos superiores y cervicales, que transmiten información de las extremidades superiores y de la región superior del tórax, ascienden en el fascículo *cuneatus*, situado lateralmente en el funículo dorsal, para terminar en el núcleo *cuneatus* localizado en la región lateral del complejo nuclear de la columna dorsal. En algunas especies de vertebrados se ha descrito el núcleo de Bischoff en la línea media de los núcleos de la columna dorsal que recibe que información de la cola (Kappers y cols., 1936).

Las aferencias primarias espinales emiten colaterales a distintos niveles rostrocaudales dentro del mismo plano parasagital de los núcleos de la columna dorsal, lo que permite establecer un mapa de representación somatotópica de la superficie corporal, que se mantiene constante en toda la extensión de los núcleos (Willis y Coggeshall, 1991).

El funículo dorsal contiene, además de las aferencias primarias, axones de neuronas situadas fundamentalmente en las láminas espinales III, IV, V y VI y en menor grado en las I, III, y VII, que proyectan a los núcleos de la columna dorsal, y constituyen el denominado sistema postsináptico de la columna dorsal. Éste participa en la transmisión de distintas modalidades de sensaciones y presenta un ordenamiento somatotópico similar al de las aferencias primarias espinales, es decir, las neuronas localizadas en los niveles cervical y lumbar proyectan a los núcleos *cuneatus* y *gracilis* respectivamente (ver Willis y Coggeshall, 1991).

En los núcleos de la columna dorsal se establece un mapa de representación somatotópico que incluye el procesamiento de información propioceptiva y exteroceptiva. En mamíferos los núcleos de la columna dorsal presentan distintas regiones citoarquitectónicas desde las que se organizan sus diferentes eferencias. Éstas incluyen una proyección descendente a la médula espinal, así como un conjunto de proyecciones ascendentes, fundamentalmente contralaterales, que constituyen el lemnisco medial. Entre las dianas diencefálicas del lemnisco medial se encuentran: la zona incerta, la región existente entre los núcleos ventrolateral y ventral posterior que recibe una proyección fundamentalmente propioceptiva, la parte medial del complejo posterior talámico, así como el núcleo ventral posterolateral del complejo ventrobasal talámico. Éste último recibe la proyección cuantitativamente más importante, relacionada fundamentalmente con la información tactil y en la cual se produce una inversión espacial del mapa de representación somática, presente en los núcleos de la columna dorsal, de manera que las proyecciones del núcleo *gracilis* terminan lateralmente a las procedentes

del núcleo *cuneatus* (ver Berkley y cols., 1986; y Willis y Coggeshall., 1991). Otras proyecciones del lemnisco medial alcanzan la región pretectal así como el núcleo rojo, colículo superior y núcleo intercolicular en el mesencéfalo; el cerebelo; y diferentes núcleos pontinos y de la oliva inferior en el rombencéfalo (ver Willis y Coggeshall, 1991).

- Otras proyecciones.

Además de las proyecciones anteriormente descritas, en relación con los núcleos de la columna dorsal; Holbrook y Wilcox (1965) observaron en el funículo dorsal de la cabra, mediante técnicas de degeneración, aferencias primarias espinales que sobrepasan rostralmente los núcleos de la columna dorsal y ascienden a través del rombencéfalo hasta el cerebelo, aunque su presencia no ha sido confirmada en trabajos posteriores en otras especies de mamíferos.

Igualmente se ha demostrado la existencia en el funículo dorsal del componente dorsal del tracto espinoreticular, que consiste en una proyección bilateral, originada principalmente en neuronas situadas en las láminas I y X y en menor medida en las láminas II-IV y VII, y que termina en el núcleo reticular dorsal (McMahon y Wall, 1985; Lima, 1990; Lima y Coimbra, 1990; Bing y cols., 1990).

Funículo dorsolateral:

- Sistema espino-cervico-talámico.

En mamíferos se ha descrito la existencia de una vía bisináptica formada por los tractos espinocervical y cervicotalámico (Morin, 1955; Nijensohn y Kerr, 1975; Boivie, 1983). El tracto espinocervical consiste en una proyección, fundamentalmente ipsilateral, que se origina en neuronas del asta dorsal situadas a lo largo de la médula espinal en el núcleo propio, láminas II, IV y V, y en niveles cervicales además en las láminas I, VI y VII. El tracto espinocervical asciende en el funículo dorsolateral y termina en el núcleo cervical lateral (Willis y Coggeshall, 1991). A lo largo de su recorrido el tracto espinocervical emite colaterales que alcanzan distintas dianas intraespinales, estableciendo conexiones intersegmentarias (Snow y cols., 1976; Rastad y cols., 1977; Jankowska y cols., 1979; Maxwell y Koerber, 1986; Cao y cols., 1993). El núcleo cervical lateral es una población neuronal propia de segmentos cervicales superiores (C1-C3), ventrolateral al asta dorsal, y segregada dentro del funículo dorsolateral. Dicho núcleo organiza proyecciones ascendentes contralaterales que alcanzan las regiones somatosensoriales mesencefálicas y talámicas, a través de los tractos cervicomesencefálico y cervicotalámico respectivamente, los cuales forman parte del lemnisco medial (ver Willis y Coggeshall, 1991). El tracto cervicomesencefálico inerva principalmente el núcleo intercolicular y las capas profundas del colículo superior, mientras que el cervicotalámico termina mayoritariamente en el núcleo ventral posterolateral y en la parte medial del complejo posterior talámico. El núcleo lateral cervical origina también proyecciones descendentes a la médula espinal (Svensson y cols., 1985b).

- Tracto espinocerebeloso dorsal.

En mamíferos el tracto espinocerebeloso dorsal o tracto de Flechsig está formado por fibras mielínicas que transmiten información sensorial propioceptiva, procedente de husos musculares y receptores tendinosos de Golgi de las extremidades posteriores, asciende en la parte dorsal del funículo dorsolateral y penetra en el cerebelo a través del cuerpo restiforme (Yoss, 1952a), para terminar en distintas regiones cerebelosas (Grant, 1962b; Manns, 1973; Matsushita y Okado, 1981; Ito, 1984). Dicha proyección se origina en neuronas de la columna de Clarke, situada en la base del asta dorsal en niveles torácicos y lumbares (hasta L4), que reciben aferencias desde los funículos dorsal y lateral así como de neuronas del asta dorsal. Sin embargo, se han descrito neuronas en niveles cervicales, que proyectan al cerebelo, cuyos axones podrían ascender a través del funículo dorsolateral (Ito, 84).

- Sistema espino-rombencefalico-talámico.

El sistema espino-rombencefálico-talámico está formado por las proyecciones de las neuronas espinales de la columna de Clarke, las cuales reciben aferencias propioceptivas, procedentes de receptores musculares de tipo Ia de los miembros posteriores. Los axones de las neuronas de la columna de Clarke ascienden en el funículo dorsolateral formando el tracto espinocerebeloso dorsal, que emite colaterales hacia la sustancia gris para sinaptar en el núcleo Z del rombencéfalo. Dicho núcleo establece proyecciones contralaterales, a través del lemnisco medial, que terminan en el núcleo ventral posterolateral así como en la región de éste que limita con el núcleo ventral lateral (Landgren y Silfvenius, 1969; 1971; Low y cols., 1986; Willis y Coggeshall, 1991).

- Otras proyecciones.

Otros tractos espinales ascendentes en el funículo dorsolateral incluyen las proyecciones espinomesencefálicas (Zelman y col., 1978; McMahon y Wall, 1983, 1985; Baker y Giesler, 1984; Swett y cols., 1985), espinohipotalámicas (Dado y cols., 1994c) y espinotalámicas (Jones y cols., 1985, 1987; Apkarian y Hodge, 1989a,b; Dado y cols., 1994c), descritas en algunos mamíferos, y que se originan en neuronas situadas en diversas regiones espinales mayoritariamente en el asta dorsal.

Funículos ventral y ventrolateral:

En mamíferos el sistema ventrolateral que ocupa los funículos ventral y ventrolateral, está formado principalmente por los tractos espinoreticular, espinocerebeloso ventral, espinomesencefálico, espinohipotalámico y espinotalámico.

- El tracto espinoreticular.

En el funículo ventrolateral existen dos tipos de proyecciones espinoreticulares. La primera se origina en neuronas de las láminas V, VII y VIII, y de manera muy escasa en la lámina X así como en el asta dorsal superficial de todos los niveles espinales, aunque más densamente desde niveles cervicales superiores (ver Willis y Coggeshall, 1991) y alcanza

fundamentalmente el núcleo reticular lateral, que a su vez proyecta al cerebelo (Oscarsson, 1973). La segunda consiste en el componente medial del tracto espinoreticular que asciende hasta diversos núcleos del tronco cerebral que proyectan a la médula espinal, al mesencéfalo o a los núcleos intralaminares del tálamo (Peschanskiy Besson, 1984; Willis y Coggeshall, 1991).

- Tracto espinocerebeloso ventral.

En mamíferos la proyección espinocerebelosa ventral o tracto de Grower está formada por fibras mielínicas que ascienden en la superficie ventrolateral de la médula espinal para entrar en el cerebelo, a través del pedúnculo cerebeloso superior (Yoss, 1952). Dicha proyección se origina en neuronas de gran tamaño, localizadas fundamentalmente en la sustancia gris dorsomedial y ventrolateral del asta ventral espinal en niveles lumbares (Cooper y Sherrington, 1940; Hubbard y Coscarsson, 1962; Lundberg y Weight, 1971; Burke y cols., 1971), que reciben información propioceptiva procedente de husos musculares y de receptores tendinosos de Golgi de las extremidades posteriores, así como de diversas proyecciones descendentes (Ito, 1984). Además, algunas fibras de las neuronas espinales que proyectan al cerebelo desde niveles cervicales, podrían igualmente ascender a través del funículo ventrolateral (Ito, 1984).

- Tracto espinomesencefálico.

Algunas proyecciones espinomesencefálicas ascienden a través del funículo dorsolateral como se ha expuesto anteriormente, sin embargo otras, esencialmente contralaterales, ascienden por el sistema ventrolateral, e incluyen proyecciones que se originan en diversas poblaciones neuronales de la médula espinal y terminan en tres regiones mesencefálicas diferenciables: 1) una zona de transición entre el rombencéfalo y el mesencéfalo, que incluye el área parabraquial, el núcleo cuneiforme y la sustancia gris periacueductal más caudal; 2) la región intermedia, que comprende el núcleo intercolicular, el núcleo cuneiforme, la sustancia gris periacueductal correspondiente a dicha zona y las capas profundas del colículo superior; y 3) niveles mesencefálicos rostrales que incluyen los núcleos de Darkschewitsch, pretectales anterior y posterior, rojo, de Edinger-Westphal e intersticial de Cajal (Yezierski, 1988). Las proyecciones espinomesencefálicas se originan en las láminas I, III, V, VII, VIII, X y en el núcleo espinal lateral, en toda la extensión rostrocaudal de la médula, así como en el núcleo cervical lateral (ver Willis y Coggeshall, 1991).

- El tracto espinohipotalámico.

En mamíferos el tracto espinohipotalámico es una proyección bilateral, aunque con predemonio contralateral, que se origina mayoritariamente en neuronas de las regiones profundas del asta dorsal, en el núcleo espinal lateral y en menor medida en el asta dorsal superficial, la sustancia intermedia y la región que rodea al canal central (Burstein y cols., 1990). En diversos trabajos se ha descrito que las proyecciones espinales alcanzan regiones mediales, laterales, ventrales y posteriores hipotalámicas, así como la región de la decusación supraóptica (Anderson y Berry, 1959; Minderhoud, 1967; Yamada y Otani, 1978; Kerr, 1975; Ju, 1984; Burstein y cols., 1987,

1990; Dado y cols., 1984a,b,c). La proyección espinohipotalámica asciende a través de los funículos ventrolateral y en menor grado dorsolateral (Dado y cols., 1994c).

- El tracto espinotalámico.

El tracto espinotalámico de mamíferos es una proyección principalmente contralateral, que se origina en neuronas situadas en todos los niveles espinales, aunque su máxima densidad corresponde a niveles cervicales superiores, donde se observa además la mayor concentración de células de proyección ipsilateral. En el resto de la médula espinal se origina mayoritariamente en los niveles de las intumescencias, cervical y en menor número de la lumbar. Se han descrito tres componentes en la proyección espinotalámica, en función de la localización de las células de origen, y de los centros de destino. 1) Constituído a patir de neuronas situadas mayoritariamente en la lámina I, aunque también en las láminas II y III, y que proyectan principalmente al núcleo ventral posterolateral y en menor grado al núcleo submedio y a los núcleos intralaminares. 2) Se organiza a partir de neuronas de las regiones profundas del asta dorsal (láminas IV-VI) y termina en los núcleos ventral posterolateral y centrolateral. 3) formados por axones de neuronas de las láminas VII-X que proyectan al núcleo centrolateral. El complejo posterior recibe igualmente proyecciones espinotalámicas (Willis Coggeshall, 1991).

Principales modalidades sensoriales y canales funcionales

Tacto-presión

El tacto y la presión constituyen dos componentes de una modalidad sensorial cuyas diferencias residen en la intensidad del estímulo. Ambos son percibidos a través de dos tipos de mecanorreceptores de adaptación lenta, SAI y SAII, que corresponden a los receptores de Merkel y de Ruffini respectivamente. Los receptores de Merkel (SAI) desempeñan un papel más importante que los de Ruffini (SAII), en esta modalidad sensorial, ya que intervienen en la transmisión de tacto y de presión, mientras que los receptores SAII producen sensaciones de presión mantenida, y están más implicados en la transmisión de información propioceptiva de sentido espacial (Harrington y Merzenich, 1970, Knibestöl y Vallvo, 1970; Willis y Coggeshall, 1991).

Las aferencias procedentes de los receptores SAI y SAII terminan en el asta dorsal en el núcleo propio y las láminas III, IV y V (Brown, 1977, Brown y cols., 1978). La información de tacto y presión es transmitida a niveles supraespinales a través de los funículos dorsal y dorsolateral.

En el funículo dorsal la información tactil, procedente de las extremidades anteriores, es transmitida por fibras primarias espinales, mientras que la de las extremidades posteriores asciende a través del sistema postsináptico de la columna dorsal. La información alcanza, por ambos sistemas, los núcleos de la columna dorsal que posteriormente la transmiten, a través del lemnisco medial, a diversas áreas mesencefálicas y talámicas.

Las fibras tactiles en el funículo dorsolateral están en relación con aferencias a los núcleos de la columna dorsal, si bien las del tracto espinocervical parecen no estar implicadas (ver Willis y Coggeshall, 1991). Willis y cols. (1974) demostraron cierto grado de implicación del cuadrante ventrolateral espinal, en la transmisión de información tactil.

Temblor-Vibración

Esta modalidad sensorial incluye varios tipos de sensaciones, principalmente el temblor y la vibración, percibidas al aplicar en la superficie corporal estímulos oscilatorios de distintas frecuencias, entre 5 y 40 Herz o superior a 60 Herz respectivamente (Willis and Coggeshall, 1991). Su percepción en mamíferos parece depender de la activación de mecanorreceptores de bajo umbral y adaptación rápida, FAI (temblor) y FAII (vibración), localizados en la piel y en tejidos subyacentes respectivamente (Limdblom y Lund, 1966), y que se distinguen entre sí morfológica y electrofisiológicamente (Willis and Coggeshall, 1991). Los receptores FAI incluyen a los copúsculos de Meissner en las regiones de piel sin pelo, y a los receptores de campo y de folículo piloso, en las regiones de piel dotadas de pelo. Los receptores FAII corresponden a los corpúsculos de Paccini (Lindblom y Lund, 1966; Knibestöl, 1973).

En los primeros experimentos, basados en técnicas microneurográficas, se sugirió la implicación de los mecanorreceptores de adaptación rápida (FAI y FAII) en la evocación de sensaciones descritas como tacto, presión, cosquilleo y vibración (Vallvo, 1981), aunque en estudios posteriores (Schady y cols., 1983; Vallvo y cols., 1984) se han relacionado únicamente con la percepción de temblor, vibración y de golpes ligeros, dependiendo de la frecuencia de la estimulación.

Igualmente se ha determinado que los mecanorreceptores tipo C en regiones restringidas de la piel (Kumazawa y Perl, 1977; Nordin, 1990), así como los receptores pacciniformes de las articulaciones y los receptores musculares (Willis y Coggeshall, 1991) pueden estar implicados en la percepción de sensaciones de cosquilleo, temblor y vibración.

Las aferencias primarias procedentes de los mecanorreceptores FAI, que evocan sensaciones de temblor, terminan en las capas profundas del asta dorsal (láminas III y IV) (Brown y cols., 1977) y algunas ascienden en el funículo dorsal hasta los núcleos de la columna dorsal (Willis y Coggeshall, 1991). Sin embargo, las aferencias procedentes de los receptores FAII terminan en las regiones mediales de las láminas III y VI (Brown y cols., 1980).

Las proyecciones ascendentes a través de las cuales los estímulos de temblor (mecanorreceptores FAI) son conducidos al complejo ventrobasal del tálamo, incluyen el sistema columna dorsal (aferencias primarias y no primarias)-lemnisco medial en el funículo dorsal, el sistema espino-cervico-talámico en el funículo dorsolateral (Brown y Franz, 1969; Bryan y cols., 1974; Willis y cols., 1974; Brown, 1981; Brown y Fyffe, 1981), y el tracto espinotalámico en el funículo ventrolateral (Willis y Coggeshall, 1991).

Los estímulos vibratorios procedentes de los receptores de Paccini (FAII), (Brown y Fyffe, 1981; Brown y cols., 1983) alcanzan el complejo ventrobasal talámico únicamente a través del sistema columna dorsal (aferencias primarias y no primarias)-lemnisco medial (Willis y Coggeshall, 1991).

Propiocepción

La propiocepción incluye el sentido de la posición y localización de los miembros en el espacio y su cinestesia, es decir, la noción de su movimiento. Dicha modalidad sensorial permite diferenciar entre movimientos activos y pasivos, así como la intensidad de la fuerza aplicada en las contracciones voluntarias (Googwin y cols., 1972; McCloskey, 1973; Horch y cols., 1975; Roland y Ladegaard-Pedersen, 1977). Existen ciertas controversias en la bibliografía acerca de los receptores encargados de la percepción de la posición (ver Willis y Coggeshall, 1991), aunque actualmente se acepta que los husos musculares están implicados en dicha función, así como en la distinción entre movimientos activos y pasivos. En mamíferos, los husos musculares poseen dos tipos de fibras aferentes: primaria y secundaria, que transmiten distintos parámetros de la contracción y el estiramiento muscular.

Los receptores de adaptación lenta de las articulaciones (terminales de Ruffini), responden ante estímulos extremos de flexión y extensión de las mismas, y están relacionados con la detección de los límites de sus movimientos, más que con la transmisión de información acerca de la posición de los miembros (Willis y Coggeshall, 1991).

Los mecanorreceptores de adaptación lenta (SAII), además de estar implicados en la trasmisión de información tactil y de presión, responden a estímulos de estiramiento de la piel y participan en la evocación de sensaciones de posición y de movimiento (Macefield y cols., 1990; Willis y Coggeshall, 1991).

Por último, los órganos tendinosos de Golgi responden ante estímulos de estiramiento y contracción muscular proporcionalmente a la fuerza de la contracción (Willis y Coggeshall, 1991).

Las aferencias musculares propioceptivas terminan principalmente en la región profunda del asta dorsal, donde se han descrito neuronas que intervienen tanto en la elaboración de reflejos motores, como en procesos sensoriales propioceptivos (Willis y Coggeshall, 1991). Las aferencias musculares propioceptivas de tipo Ia, terminan en las láminas VI, VII y IX (Brown y Fyffe, 1978, Brown, 1981), y las de tipo II en las láminas IV, VII y IX (Fyffe, 1979; Brown, 1981). Las aferencias de tipo Ib, procedentes de los órganos tendinosos de Golgi, se arborizan en las láminas VI y VII. Otras aferencias musculares que parecen ser de naturaleza nociceptiva, concluyen en las láminas I y V, (Mense y Prabhakar, 1986), en la columna de Clarke y algunas ascienden en el funículo dorsal hasta el núcleo *cuneatus* externo (Craig y Mense, 1983; Nyberg y Blomqvist, 1984; Hongo y cols., 1987).

La información propioceptiva, procedente de los mecanorreceptores del músculo y de las articulaciones de las extremidades anteriores, asciende a través del fascículo *cuneatus* de la columna dorsal

hasta el núcleo *cuneatus* (Willis y Coggeshall, 1991). Sin embargo, las aferencias propioceptivas de las extremidades posteriores (Lloyd y McIntyre, 1950; Burguess y Clark, 1969), ascienden a través del sistema espino-rombencefalico-talámico, primero en el funículo dorsolateral hasta el núcleo Z rombencefálico, el cual proyecta al tálamo contralateral (Willis y Coggeshall, 1991). Aunque no existen evidencias definitivas, se ha sugerido la participación del tracto espinotalámico en la propiocepción (Willis, 1974; Appelbaum y cols., 1975; Milne y cols, 1982).

Dolor

La percepción del dolor incluye dos tipos fundamentales de sensación de acuerdo con Lewis (1942): superficial y profundo. El dolor superficial es una sensación localizada, resultado de la estimulación de la piel, que puede ser dividido en primario y secundario, transmitidos a través de fibras A delta y C respectivamente, que evocan dos sensaciones dolorosas sucedidas en el tiempo, debido a la velocidad diferencial de transmisión de ambos tipos de fibras (Lewis y Pochin 1938; Lewis, 1942; Sinclair y Strokes, 1964; Price y cols., 1977; Campbell y LaMotte, 1983). El dolor profundo es una sensación con una localización imprecisa que se origina en tejidos subcutáneos como el músculo esquelético, tendones, periostio, y articulaciones. Un tercer tipo de dolor es el visceral, que comparte diversas características con el dolor profundo. Una de las consecuencias de las sensaciones dolorosas consiste en provocar respuestas en el sistema nervioso autónomo, cambios endocrinos y respuestas motivacionales y afectivas (ver Willis y Coggeshall, 1991). Por último se ha descrito una modalidad de dolor neurogénico que se produce sin una necesaria activación de los nociceptores y está asiociado con lesiones del sistema nervioso central (ver Willis y Coggeshall, 1991).

Los nociceptores, receptores encargados de percibir los estímulos nociceptivos, consisten en fibras con terminaciones libres no encapsuladas, escasamente mielinizadas (A delta) o amielínicas (C), que transmiten el estímulo con una mayor o menor velocidad respectivamente (Burgess y Perl, 1967; Georgopoulos, 1976; Willer y Albe-Fessarrd, 1983). No existen diferencias estructurales, entre los que inervan distintos órganos o tejidos (piel, músculo, articulaciones o visceras). Se han desrito, sin embargo, varios tipos funcionales de nociceptotes, dependiendo de parámetros fisiológicos como el umbral de activación, o el estímulo o estímulos necesarios para su excitación. En la piel, por ejemplo, están presentes diversos nociceptores A delta que se activan ante estímulos mecánicos, de frio, o a ambos; nociceptores C que responden igualmente a estímulos de frio, mecánicos y térmicos, y receptores polimodales que se excitan frente a estimulación mecánica, térmica y química.

Las aferencias primarias de los nociceptores A delta terminan en las láminas I y V (Light y Perl, 1979), mientras que las procedentes de los nociceptores C se arborizan en las láminas I y II (Sugiura y cols., 1986). En las láminas I, II y V se han descrito neuronas que responden a estimulación nociceptiva, así como en los núcleos de la columna dorsal que proyectan contralateralmente al tálamo. La información nociceptiva que llega a los núcleos de la

columna dorsal, asciende a través del sistema postsináptico de la columna dorsal (Angaut Petit 1975a,b; Giesler y Cliffer, 1985; Brown y cols., 1983; Bennett y cols., 1984; Kamogawa y Bennet, 1986) y en menor medida a través de las aferencias primarias.

En el funículo dorsolateral, algunas fibras de los tractos espinocervical (Brown y Franz, 1969; Bryan y cols., 1973, 1974; Cerveró y cols., 1977) y espinotalámico dorsal (Apkarian y Hodge a,b, 1989) responden a estimulación nociceptiva.

La mayoría de las neuronas de la proyección espinotalámica, en el funículo ventrolateral, responden a estimulación nociceptiva (Willis y cols., 1974; Price y cols., 1978; Chung y cols., 1979; Kenshalo y cols., 1979; Surmeier y cols., 1988) aunque en ocasiones integran los estímulos dolorosos con otros tipos de información (Willis y Coggeshall, 1991). Igualmente, se ha descrito la transmisión de estímulos nociceptivos a través de los tractos espinoreticular (Fields y cols., 1975, 1977b), espinomesencefálico (Menetrey y cols., 1980) y espinohipotalámico (Willis y Coggeshall, 1991), si bien dichas proyecciones podrían estar implicadas más en los componentes afectivos y motivacionales del dolor, que en el sensorial (Willis y Coggeshall, 1991).

Temperatura

Según los resultados de los trabajos de Hensel (1950, 1973a, 1974), las sensaciones térmicas de frio y de calor se producen ante la variación ascendente o descendente de la temperatura neutra de la piel, estimada en 32-37°C. Los extremos en la sensación térmica, tanto de frio (Wolf y Hardy, 1941) como de calor (Hardy y cols., 1951; Neisser, 1959; LaMotte y Campbell, 1978) evocan sensaciones dolorosas.

La sensación térmica depende de la activación de los receptores específicos de frio y de calor. Los primeros están innervados por fibras A delta y C, mientras que los de calor, únicamente reciben fibras C (ver Willis y Coggeshall, 1991) y responden a variaciones de temperatura de la piel, y a determinados agentes químicos (Hensel y Zotterman, 1951c; Dodt y cols., 1953; Hensel, 1973a) que provocan sensaciones térmicas. Las sensaciones de frio pueden además ser producidas por la disminución en la actividad de los receptores de calor, causada por un descenso de la temperatura, aunque no existen evidencias del fenómeno contrario. Las variaciones térmicas afectan a la actividad de algunos mecanorreceptores, si bien este fenómeno no parece intervenir significativamente en el origen de sensaciones térmicas (Willis y Coggeshall., 1991).

Las aferencias cutáneas A delta y C, que probablemente incluyen las procedentes de receptores térmicos, terminan en las láminas I, II y V (Cerveró, 1989) en las que se han descrito neuronas que responden a la estimulación térmica (Willis y Coggeshall, 1991). Hasta el momento, únicamente se ha demostrado la implicación de las proyecciones espinotalámicas, originadas en neuronas de lámina I y que ascienden en el sistema ventrolateral, en la transmisión de información térmica a centros supraespinales (Craig y Kniffki, 1985; Ferrington y

cols., 1987, Kanui, 1988). Algunas de las neuronas que originan esta proyección, responden exclusivamente a información térmica (Craig y Kniffki, 1985), aunque en otras se produce una integración con otras estimulaciones generalmente nociceptivas (Craig y Kniffki, 1985; White y Sweet, 1969). Foerster y Gagel (1932) describieron que los axones de las neuronas espinotalámicas termorreceptivas parecen ascender en el funículo ventrolateral, segregados dorsalmente respecto a las proyecciones nociceptivas. En 1985 Craig y Kniffki demostraron que la proyección espinotalámica termoreceptiva alcanza la parte medial del complejo ventrobasal talámico, donde se han descrito neuronas que responden a variaciones térmicas de la piel de distintas regiones corporales (ver Willis y Coggeshall, 1991).

Sensaciones viscerales

Las percepciones viscerales incluyen la activación de receptores que transmiten la información necesaria para la regulación del medio interno, y no provocan sensaciones conscientes, como la información procedente de los barorreceptores arteriales, o de los quimiorreceptores de los cuerpo carotídeos (Cerveró y Foreman, 1990). Por el contrario, existen receptores que transmiten información visceral que sí evoca sensaciones conscientes. La sensación visceral mayoritaria es el dolor, si bien existen otras, como la de saciedad o el sentido de vaciamiento de los órganos (Leek, 1972). Aunque se ha descrito la existencia de termorreceptores abdominales (Riedel, 1976), estos deben intervenir en la termorregulación corporal, ya que aparentemente no se producen sensaciones térmicas viscerales.

La inervación sensorial de las vísceras es cuantitativamente menor que la de la piel (Cerveró y Foreman, 1990), sin embargo, presenta una dualidad ya que los órganos internos están inervados por el sistema nervioso simpático y el parasimpático (ver Cerveró y Foreman, 1990). En general la sensación visceral de dolor es percibida a través del sistema nervioso simpático el cual proyecta al sistema nervioso central a través de las raíces dorsales espinales. Por el contrario, la entrada de otros tipos de información viceral, requeridos para la elaboración de reflejos y para la regulación visceral, es percibida a través del sistema nervioso parasimpático, y alcanza el sistema nervioso central a través de los pares craneales VII, IX y X (Cerveró y Foreman, 1990).

El 90% de los receptores viscerales son fibras amielínicas, o fibras que pierden la mielina al penetrar en la víscera (Kuo y cols., 1982). Se han descrito nociceptores viscerales en el corazón, los pulmones, la vejiga urinaria, los conductos biliares, los testículos y en el útero (Cerveró, 1985). Igualmente se han observado receptores en el mesenterio y en la vaina serosa de diversos órganos, que se activan ante estímulos de movimiento y distensión de los mismos, y por lo tanto podrían contribuir a las sensaciones de saciedad y de dolor en aquellos casos de estiramientos excesivos (Willis y Coggeshall, 1991). Asimismo, se han descrito receptores en el músculo liso del tracto gastrointestinal y de la vejiga, que parecen intervenir en la sensación de vaciamiento de los distintos órganos, así como en el dolor (Leek, 1972), ya que

responden a estímulos de distensión y de contración (Winter, 1971, Leek, 1972).

Las aferencias viscerales primarias mielínicas terminan en las láminas I, II, V-VII y X espinales sacras. A nivel torácico terminan en las láminas I y V y con menor densidad en la parte externa de la lámina II. Sugiura y cols. (1989) han descrito que las aferencias de las fibras amielínicas terminan en las láminas I, II, IV-V y X (Cerveró y Foreman, 1990; Willis y Coggeshall, 1991). Algunas de las aferencias viscerales ascienden y descienden a niveles espinales adyacentes a través del tracto de Lissauer en el funículo dorsolateral (Cerveró y Foreman, 1990).

La información visceral asciende a niveles supraespinales por el funículo dorsal hasta los núcleos de la columna dorsal (Amassian, 1951; Aidar y cols., 1952; Perl y cols., 1962). Asimismo se ha descrito la existencia de fibras ascendentes viscerales en el funículo dorsolateral, procedentes de neuronas del asta dorsal superficial (Cerveró y Foreman, 1990). Sin embargo, las proyecciones espinoreticular y espinotalámica del sistema ventrolateral constituyen cuantitativamente los sustratos anatómicos más importantes, por los cuales asciende la información visceral (Cerveró y Foreman, 1990; Willis y Coggeshall, 1991). Se ha sugerido que las proyecciones espinosolitarias, que se originan en neuronas de las láminas I, V, X y en el núcleo espinal lateral (Menetrey y Basbaum, 1987; Leha y cols., 1988); y las proyecciones espinomesencefálicas que terminan en la sustancia gris central y en el área parabraquial, podrían también estar implicadas en procesos de integración viscerosomáticos y visceroviscerales (Cerveró y Foreman, 1990).

VERTEBRADOS NO MAMÍFEROS

Dentro de los vertebrados no mamíferos el estudio del sistema nervioso de los anfibios resulta de gran interés desde el punto de vista evolutivo, por representar éstos un grupo taxonómico intermedio entre los vertebrados acuáticos y los terrestres.

Los anfibios surgieron en el periodo Devónico superior, a partir de los peces Crosopterigios, y sufrieron numerosas adaptaciones motivadas por la transición que experimentaron al cambiar del hábitat acuático al terrestre. Algunas de estas transformaciones se producen durante la ontogenia de los anfibios actuales, debido a que a lo largo de su desarrollo embrionario y larvario experimentan un cambio en el modo de vida, que en estadios tempranos se desarrolla estrictamente en el agua y pasa posteriormente a depender de forma relativa de los hábitats acuáticos.

Actualmente los anfibios están clasificados en tres órdenes: *Anura, Urodela y Apoda.* Cada uno de ellos incluye géneros con formas de vida que se desarrollan con un grado variable de dependencia del agua. Todos ellos presentan características comunes con los vertebrados acuáticos, así como con los terrestres.

Las transformaciones producidas a lo largo de la ontogenia de los anfibios, incluyen entre otras, la regresión de las branquias externas, la adquisición de pulmones saculares que permiten el aprovechamiento del oxígeno atmosférico, las variaciones

tegumentarias que impiden parcialmente la desecación, el desarrollo de las extremidades y las variaciones en el aparato locomotor como los cambios del esqueleto y de las masas musculares necesarios para la adquisición de los distintos patrones de locomoción propios de cada grupo. De forma paralela a dichas transformaciones, los anfibios sufren variaciones en los sistemas sensoriales y en el sistema nervioso central. Se produce una regresión o involución del sistema de la línea lateral, presente en los anamniotas acuáticos, en los géneros cuyos individuos adultos abandonan el medio acuático (Wahnschafre y cols., 1987). Una transformación sorprendente durante la ontogenia de algunos géneros de anfibios es la producida al cambiar de un modelo de locomoción natatoria, mediante movimientos corporales cuyo control nervioso reside básicamente en la médula espinal (Hughes, 1957; Roberts y cols., 1983), a un modelo de locomoción tetrápodo, coordinado por un control nervioso supraespinal. Paralelamente se producen variaciones en los sistemas sensoriales espinales en los que el sistema de Rohon-Beard es progresivamente reemplazado por el desarrollo del sistema constituído por las células ganglionares de las raíces dorsales (Forehand y Farel, 1982; ten Donkelaar y de Boer-van Huizen, 1991).

Receptores periféricos somatosensoriales en los anfibios

En los anfibios se han descrito distintos tipos de receptores cutáneos responsables de la recepción de estímulos somatosensoriales, que incluyen tacto, presión, temperatura y dolor. Se diferencian cuatro tipos de receptores cutáneos, atendiendo a diversos parámetros morfológicos y fisiológicos como el diámetro de la fibra, el modo de terminación libre o encapsulado, la distribución dérmica o epidérmica, el tipo de estímulo percibido, así como la velocidad de transmisión del estímulo y la adaptación rápida o lenta del receptor (Marushashi y cols., 1952; Lindblon, 1962; Spray, 1976). En general, los mecanorreceptores consisten en terminaciones relativamente gruesas, libres o encapsuladas que se distribuyen en la epidermis, o en la zona de transición con la dermis y tienen una alta velocidad de conducción. Los receptores que responden a estímulos térmicos, y los que lo hacen frente a estímulos nociceptivos corresponden a fibras de menor diámetro, de terminación libre, que generalmente se distribuyen en las regiones profundas de la dermis, con una menor velocidad de conducción que en el caso de los mecanorreceptores. Dentro de los mecanorreceptores, Catton (1958, 1976) diferenció entre los que responden a estímulos tactiles o a presión, así como cuatro tipos diferentes de mecanorreceptores en base a parámetros fisiológicos carentes de diferencias morfológicas aparentes.

Las aferencias propioceptivas musculares provienen en los anfibios de los husos musculares, órganos encapsulados que responden a estímulos provocados por variaciones de tensión y de estiramiento de su fibra muscular (Katz, 1961; Ottoson, 1976). Los husos musculares de la rana tienen un único tipo de terminal sensitivo, homólogo del primario de los mamíferos, consistente en una fibra mielínica que penetra en la cápsula y progresivamente va perdiendo la mielina al ramificarse sobre la fibra intrafusal (Barker, 1974; Ottoson, 1976).

La médula espinal de los anfibios

La médula espinal de los anuros únicamente posee 11 segmentos y presenta dos intumescencias, una cervical (o braquial) y otra lumbar que corresponden a los niveles de inervación de las extremidades anteriores y posteriores respectivamente. Después de la metamorfosis, se observan diez nervios espinales que se numeran del 2 al 11, excluyendo el primer nervio ya que está muy poco desarrollado (Gaupp, 1899; Hughes y Tschumi, 1958; Deuchar, 1975; Thors, 1980). Dicha médula espinal está conectada con cada uno de los diez de pares de nervios espinales por medio de una raíz dorsal y otra ventral. En el género *Rana* la raíz ventral del segundo nervio espinal constituye el nervio hipogloso que, en parte, se incorpora al plexo braquial. En *Xenopus laevis* la raíz ventral del segundo nervio espinal únicamente contribuye a la formación del plexo braquial (Deuchar, 1975), mientras que dicho nervio carece del ganglio de la raíz dorsal. Los nervios 3 y 4 forman el plexo braquial e inervan las extremidades anteriores, los nervios espinales 5, 6 y 7 inervan el tronco, mientras que los 8, 9 y 10 forman el plexo lumbosacro que inerva las extremidades posteriores. El polo caudal de la médula o *filum terminale*, se reduce en los anuros a un cono relativamente largo, compuesto por un canal central, rodeado por una población celular mayoritariamente glial, que representan los restos de la involución sufrida por esta región durante la metamorfosis, al desaparecer la musculatura de la cola a la cual inervaba. No parecen existir excesivas diferencias interespecíficas en anuros en cuanto a la organización básica de la médula espinal, excepto en la variación de su longitud (Ebbesson, 1976).

En los anfibios urodelos la médula espinal se extiende a lo largo de toda la longitud del canal vertebral. Como en el caso de los anuros, se distinguen dos intumescencias, cervical o braquial y lumbar, que representan las regiones de inervación de las extremidades. Nuestro conocimiento de la médula espinal de los urodelos es incompleto, debido a la escasez de trabajos experimentales de trazado neuronal, que se limitan a estudios sobre las proyecciones de las raíces dorsales, algunas vías espinales ascendentes, conexiones propioespinales, así como sobre el recorrido y el lugar de terminación de algunas vías supraespinales descendentes.

Las secciones transversales de la médula espinal en anuros, a diferencia de la de urodelos que presenta una morfología ovalada, muestran la típica forma de H de la sustancia gris, presente en la mayoría de los grupos de vertebrados terrestres. Las astas dorsales, relativamente pequeñas en anuros, están separadas mutuamente por los funículos dorsales. Las astas ventrales poseen grupos de motoneuronas organizadas de manera relativamente simple, (ver Cruce, 1974; Frank y Westerfield, 1982; Hulshof y cols., 1987) que dan origen a las raíces ventrales espinales.

- Citoarquitectura

Debido a la escasa diferenciación de sus diversos grupos celulares, la sustancia gris espinal de anfibios se ha subdividido, en base a los resultados obtenidos en estudios con técnicas de Golgi, en una serie de campos espinales, coincidiendo con los lugares de terminación de los sistemas de fibras aferentes (Ebbesson, 1976), que contrasta con la subdivisión en láminas, citoarquitectónicamente

diferenciables, establecida en mamíferos (Rexed 1952). Ebbesson (1976) en los anuros *Rana catesbeiana* y *R. pipiens,* ha definido los campos dorsal, lateral, central, ventrolateral y ventromedial, así como los campos motor lateral, que inerva la musculatura de las extremidades, y motor medial que inerva la musculatura axial. Esta subdivisión es extensible a otras especies de anuros como *R. esculenta* o *Xenopus laevis* (Nikundiwe y cols. 1982).

El *campo dorsal* en anuros ocupa la mayor parte del asta dorsal, y es comparable a las láminas I a IV de Rexed (1964) de mamíferos, aunque sus límites son difíciles de establecer (Ebbesson, 1976). No obstante, las fibras de las raíces dorsales forman un plexo terminal característico sobre la superficie del asta dorsal, que fue descrito por Silver (1942) como el *neuropilo dorsal,* formado principalmente por fibras aferentes de las raíces dorsales y, en los primeros segmentos espinales, por aferencias primarias del nervio trigémino (Fuller y Ebbesson, 1973; Matesz y Székely, 1978; González y cols., 1993). En el campo dorsal se han descrito distintos tipos neuronales con prolongaciones dirigidas al neuropilo dorsal, a los funículos dorsal y dorsolateral, así como al neuropilo lateral (Ebbesson, 1976).

El *campo lateral* recibe fibras de las raíces dorsales (*neuropilo ventral*), que determinan sus límites medial y ventral (Joseph y Whitlock 1968b; Ebbesson, 1976). Además le llegan dorsalmente fibras del tracto reticuloespinal (Mensah, 1974), así como fibras del tracto rubroespinal (Corvaja y Grofová, 1972; ten Donkelaar 1982, 1992; Larson-Prior y Cruce, 1992); y carece de aferencias trigeminales (Fuller y Ebbesson, 1973; Matesz y Székely, 1978; González y cols., 1993). En este campo espinal existen dos tipos celulares, en la región medial se han descrito neuronas pequeñas con dendritas cortas y restringidas a los límites del campo, mientras que en las regiones laterales existen neuronas de mayor tamaño con dendritas dirigidas hacia todos los funículos. Debido al patrón de sus aferencias, Ebbesson (1976) comparó el campo lateral de los anuros con las láminas V-VI de Rexed (1964) en mamíferos.

El *campo motor lateral,* presente en los niveles de las intumescencias espinales, está formado por motoneuronas que se organizan en un grupo o columna lateral, el cual inerva la musculatura de las extremidades. Mientras que el *campo motor medial,* observable en todos los niveles espinales, está constituído por una columna delgada de motoneuronas más mediales que inervan la musculatura axial (Cruce, 1974; Frank y Westerfield, 1982; Hulshof y cols., 1987).

Los *campos ventrolateral* y *ventromedial* se caracterizan por sus aferencias supraspinales (Corvaja y Grofová, 1972; Corvaja y cols., 1973; Fuller, 1974; Mensah, 1974; ten Donkelaar, 1982). Ebbesson (1976) comparó los campos ventrolateral y ventromedial con las láminas VII y VIII de Rexed (1964) respectivamente. El *campo ventrolateral* está probablemente inervado por fibras reticulospinales, pero no recibe aferencias vestibulospinales y tectospinales (Ebbesson, 1976). Las neuronas de dicho campo son pequeñas o medianas y existen al menos tres tipos de acuerdo con su morfología dendrítica (Ebbesson, 1976). El *campo ventromedial* es la zona de terminación prioritaria para las

proyecciones tectoespinales (Rubinson, 1968) y vestibuloespinales (Corvaja y Grofová, 1972; Corvaja y cols., 1973; Fuller, 1974), si bien también culminan en él algunas fibras reticuloespinales (Mensah, 74). Sus neuronas poseen árboles dendríticos reducidos y dirigidos bilateralmente al funículo ventral (Ebbesson, 1976).

El *campo central,* localizado alrededor el canal central, es comparable a la lámina X de Rexed (1964), y está compuesto por dos tipos neuronales (Ebbesson, 1976) según dirijan sus dendritas a todos los campos adyacentes o a los campos central y dorsal.

En urodelos los datos existentes sobre la organización citoarquitectónica de la médula espinal, se basan únicamente en la escasa información resultante de estudios pioneros (Burckhardt, 1889; Studnicka, 1895; Van Gehuchten, 1897; Nieuwenhuys, 1964). Las *astas dorsales* están formadas fundamentalmente por células pequeñas que constituyen la sustancia gelatinosa (Nieuwenhuys, 1964), aunque Burckhardt (1889) y Studnicka (1895) describieron algunas neuronas más grandes. La *zona intermedia* de la sustancia gris, situada ventralmente a la sustancia gelatinosa y dorsalmente a las motoneuronas, está formada por células pequeñas y medianas que extienden sus dendritas casi exclusivamente a la sustancia blanca (Van Gehuchten, 1897). Las *astas ventrales* son una masa compacta que contiene numerosas neuronas grandes que proyectan ventrolateralmente (Van Gehuchten, 1897).

- Patrón de terminación espinal de las proyecciones de las raíces dorsales

Estudios basados en técnicas de degeneración anterógrada (Liu y Chambers 1957; Joseph y Whitlock, 1968a,b; Liu, 1969) sugirieron que las fibras de las raíces dorsales terminan fundamentalmente en el asta dorsal, y no en el asta ventral. Igualmente se demostró que ascienden hasta niveles del tronco cerebral, y que poseen ramas descendentes que se extienden hasta cinco y seis segmentos caudalmente al nivel de la raíz lesionada. Joseph y Whitlock (1968a,b) propusieron que las neuronas del asta ventral, probablemente recibían proyecciones de fibras de las raíces dorsales a través de sus dendritas que invaden el asta dorsal. Sin embargo, la aplicación de técnicas de marcaje con cobalto en *Rana esculenta* (Székely, 1976; Antal y cols., 1980; Székely y cols., 1982; Székely y Antal, 1984) y con HRP en *Rana catesbeiana* (Frank y Westerfield, 1982; Jhaveri y Frank, 1983; Liuzzi y cols., 1984; Lichtman y cols., 1984; Smith y Frank, 1988a), *Rana pipiens* (Rosenthal y Cruce, 1985), *Rana ridibunda* (Motorina y cols., 1982a,b; Grantyn y cols. 1982, 1984a,b; Shapovalov y Shiriaev 1984a) y *Xenopus laevis* (Nikundiwe y cols., 1982; Shiriaev y Shupliakov, 1986) ha proporcionado más información sobre la organización de las aferencias primarias espinales en los anfibios anuros.

Las fibras primarias, al entrar en la médula espinal de anuros, se distribuyen segregándose en un *componente medial* de fibras de mayor diámetro, que ingresa en el funículo dorsal, para ascender hasta el núcleo de la columna dorsal y otras estructuras del tronco cerebral; y un *componente lateral* formado por axones de menor diámetro que se agrupan ventral y lateralmente a la entrada de la raíz para formar el tracto

de Lissauer, situado en el funículo dorsolateral (Székely, 1976; Rosenthal y Cruce, 1985), el cual se condidera implicado en la transmisión de información nociceptiva (Maruhashi y cols., 1952). Dichas aferencias primarias ocupan zonas espinales concretas de terminación dependiendo del origen y modalidad axonal (ver Székely y cols., 1982; Jhaveri y Frank, 1983; Grantyn y cols., 1984a,b; Székely y Antal, 1984). Posteriormente a su entrada en la médula espinal, a través de las distintas raíces, las fibras primarias del componente medial se bifurcan para dar ramas ascendentes y descendentes que discurren a través del funículo dorsal. A intervalos a lo largo de su recorrido espinal, las fibras emiten colaterales que se arborizan en la sustancia gris espinal, formando fundamentalmente dos neuropilos o campos de terminación: el *neuropilo dorsal* localizado en el asta dorsal, y el *neuropilo ventral* que ocupa el campo lateral (Székely, 1976; Frank y Westerfield, 1982; Jhaveri y Frank, 1983). Los axones procedentes de aferencias primarias cutáneas, proyectan únicamente al neuropilo dorsal (Jhaveri y Frank, 1983; Székely y Antal, 1984), mientras que la gran mayoría de las aferencias sensoriales procedentes del músculo, proyectan al neuropilo ventral, en el que se distribuyen igualmente las dendritas de motoneuronas y de interneuronas.

En segmentos cervicales son relativamente pocos los axones que profundizan ventral y lateralmente para alcanzar los somas de las motoneuronas (Frank y Westerfield, 1982; Jhaveri y Frank 1983; Lichtman y cols. ,1984; Székely y Antal, 1984). Se ha demostrado en estudios fisiológicos (Frank y Westerfield, 1982; Lichtman y Frank, 1984), que las motoneuronas del triceps braquial reciben una activación monosináptica, desde las fibras primarias con información del propio músculo. Estos datos, conjuntamente con la escasez de contactos axosomáticos demostrados morfológicamente, indican que la mayoría de las conexiones monosinápticas en la médula espinal a nivel braquial, debe producirse a través de contactos axodendríticos en el neuropilo ventral.

En segmentos torácicos el neuropilo dorsal está muy desarrollado, mientras que el ventral contiene menor número de fibras (Székely, 1976; Jhaveri y Frank, 1983; Smith y Frank, 1988a,b). Esto coincide con los experimentos mediante estimulación de las raíces dorsales en *Rana ridibunda*, (Shapovalov y Shiriaev, 1984) y en *Rana pipiens* (Carlsen y Mendell, 1977) que demostraron que las interacciones sinápticas con las motoneuronas torácicas están mediadas por interneuronas, y no se producen sinapsis sensorimotoras directas.

En segmentos lumbares las aferencias primarias establecen diversas sinapsis de paso sobre las dendritas dorsales de las motoneuronas de las regiones dorsal (Corvaja y Pellegrini, 1975; Sotelo y Grofová, 1976; Adanina y Shapovalov, 1983) y dorsolateral (Székely, 1976; Liuzzi y cols., 1984) del campo motor lateral.

En urodelos las aferencias de las raíces dorsales presentan menor diferenciación en componentes *medial* y *lateral*. La mayoría de las fibras de las raíces dorsales se bifurcan, inmediatamente después de su entrada en la médula, en ramas ascendentes y descendentes (Ramón y Cajal, 1909; Roth y Wake, 1985). Las ramas ascendentes y

mielinizadas ocupan la mayoría del funículo dorsal (Ramón y Cajal, 1909; Herrick, 1948). Mientras que la división lateral, que incluye fibras mielínicas y amielínicas, forma un fascículo comparable al tracto de Lissauer de otros vertebrados (Ramón y Cajal, 1909; Ariëns Kappers y cols., 1936; Roth y Wake, 1985). En preparaciones basadas en técnicas de Golgi, Ramón y Cajal (1909) observó en *Pleurodeles waltl* que las proyecciones de las raíces dorsales en la médula de urodelos son, aunque mucho más simples, similares a las de los anuros, y en ocasiones alcanzan posiciones próximas a las motoneuronas. En urodelos, a excepción de lo anteriormente descrito, apenas existen datos experimentales disponibles sobre las proyecciones de las fibras de las raíces dorsales. Sin embargo, en un estudio más reciente (Holder y cols., 1991) en *Ambystoma mexicanum* se demostró, que las aferencias primarias espinales se entremezclan con las dendritas dorsales de las motoneuronas que inervan las extremidades, pero no con las que inervan la musculatura axial.

Proyecciones espinales ascendentes

Estudios basados en técnicas de degeneración anterógrada (Kuru, 1956; Ebbesson, 1969, 1976, en *Rana catesbeiana*; Hayle, 1973a,b, en *Rana. temporaria*) han demostrado la existencia en anuros de dos sistemas de proyecciones espinales ascendentes: 1) sistema de aferencias *primarias* que asciende a través del funículo dorsal, hasta el núcleo de la columna dorsal, y 2) sistema de aferencias *secundarias* que asciende a través del funículo lateral, que fue descrita como el lemnisco espinal, y alcanza la formación reticular, y el techo mesencefálico. Mediante la utilización de marcaje con cobalto (Antal y cols., 1980; Urbán y Székely, 1982, *Rana. esculenta*) y de trazado neuronal con HRP (Nikundiwe y cols., 1982, *Xenopus laevis*), se ha demostrado la existencia de un sistema bien desarrollado de aferencias primarias espinales, ordenadas somatotópicamente, que ascienden, en el funículo dorsal hasta el núcleo de la columna dorsal. Esta proyección continúa rostralmente para inervar el complejo vestibular, así como la capa granular del cerebelo (tracto espinocerebeloso dorsal de Ebbesson) (Antal y cols., 1980; Székely y cols., 1980). Dichas fibras se originan en los ganglios espinales que inervan las extremidades (Antal y cols., 1980; González y cols., 1984).

Funículo dorsal:

- Sistema columna dorsal-lemnisco medial y otras proyecciones incluídas en el funículo dorsal.

La existencia del sistema columna dorsal-lemnisco medial es una característica común en el cerebro de vertebrados amniotas. Tanto en aves (Sinn, 1913; Craigie, 1928; Wild, 1985, 1989), como en reptiles (Kruger y Witkovsky, 1961; Goldby y Robinson, 1962; Ebbesson, 1967, 1969, 1978; Joseph y Withlock, 1968b, Künzle y Woodson, 1983; Pritz, 1983; Belekhova y cols., 1985; Pritz y Stritzel, 1986, 1989; Siemen y Künzle, 1994a,b), existe un patrón común de organización en cuanto a las aferencias primarias y no primarias (Funke, 1988 en aves; Pritz and Stritzel, 1994 en reptiles) de los núcleos de la columna dorsal, así como en sus proyecciones eferentes rombencefálicas,

mesencefálicas y talámicas. En anfibios exiten evidencias de una organización similar del sistema columna dorsal-lemnisco medial, lo que ha hecho sugerir a Willis y Coggeshall (1991) que su presencia es un caracter propio de vertebrados terrestres, al no haber sido descrito en los distintos grupos de peces (Zeehandelaar, 1921; Hayle, 1973), los cuales perciben gran parte de la información del medio que les rodea por el sistema de la línea lateral. No obstante, en diversas especies tanto en agnatos, petromizóntidos y mixinoideos (Northcutt y Ebbesson, 1980; Ronan y Northcutt, 1990) como en condrictios (Ebbesson y Hodde, 1981) y en osteictios (Finger, 1978; Oka et al., 1986; ver Ronan y Northcutt, 1990) se han descrito, en la región alar a nivel del óbex, terminales de proyecciones espinales que ascienden en el funículo dorsal. Sin embargo, no se han realizado hasta el momento estudios detallados de trazado neuronal anterógrado sobre las aferencias primarias espinales o de trazado retrógrado, con aplicaciones en el mesencéfalo y en el tálamo, que permitan establecer definitivamente la presencia del sistema columna dorsal-lemnisco medial en dichos vertebrados. Algunas especies de anfibios, en los que se ha sugerido la presencia del citado sistema, presentan modos de vida estrictamente acuáticos y mantienen el sistema de la línea lateral en estado adulto. Los estudios realizados hasta el momento en anfibios están mayoritariamente basados en técnicas anatómicas clásicas y degenerativas, lo que hace necesaria la aplicación de técnicas más recientes de trazado neuronal así como inmunocitoquímicas, con objeto de estudiar la organización detallada del sistema y establecer posibles homologías con otros grupos de vertebrados.

Tanto en agnatos (Northcutt y Ebbesson, 1980; Ronan y Northcutt 1990) como en condrictios (Ebbesson y Hodde, 1981), teleósteos (Ronan y Northcutt 1990) y reptiles (Ebbesson, 1967,1969; Jacobs, 1968; Pedersen, 1973; Künzle, 1982, Pritz y Stritzel, 1994), se ha descrito un componente de aferencias espinales primarias que asciende en el funículo dorsal, sobrepasa rostralmente los núcleos de la columna dorsal y llega hasta el cerebelo.

En anfibios anuros no se ha descrito el núcleo de columna dorsal *(nucleus funiculis dorsalis)* como entidad citoarquitectónica (Ariëns Kappers y Hammer, 1918; Zeehandelaar, 1921; Opdam y cols., 1976). Asimismo, Ariëns Kappers y cols. (1936), negaron la presencia en anfibios del sistema columna dorsal-lemnisco medial. Sin embargo, Woodburne (1939), en ránidos, mediante lesiones de los ganglios de las raíces lumbares, presentó evidencias de la presencia del núcleo de la columna dorsal, ya que observó la degeneración producida en una pequeña región rombencefálica situada dorsomedialmente a nivel del óbex, que definió como un primordio del núcleo *gracilis*. En trabajos basados en técnicas de degeneración anterógrada y de trazado neuronal con leucina tritiada (Liu y Chambers, 1957; Joseph y Whitlock, 1968; Ebbesson, 1969; Hayle, 1973b), se ha descrito el núcleo de la columna dorsal de los anuros como el lugar de terminación de fibras del funículo dorsal, pero sin considerarlo como una entidad citoarquitectónica. Sin embargo, Fernández de Molina y cols. (1966) y Silvey y cols. (1974) determinaron la presencia de un pequeño grupo de neuronas bien definido, ubicado en el polo rostral de la columna dorsal que responde a estimulación somatosensorial. La mayor parte de las aferencias

primarias espinales terminan en la región del núcleo de la columna dorsal, si bien un pequeño componente, al menos en el caso de las aferencias braquiales, continúa rostralmente a través del rombencéfalo para terminar en el cerebelo (Joseph y Witlock, 1968; Rushmer, 1970; Rushmer y Woodward, 1971; Székely y cols., 1980; Antal y cols., 1980; Nikundiwe y cols., 1982).

La existencia del lemnisco medial en anuros ha sido motivo de controversia hasta el principio de la década de los 80. Diversos trabajos (Vesselkin y cols., 1971; Vesselkin y Kovacevic, 1973; Silvey y cols., 1974; Neary y Wilczynski, 1977) propusieron la presencia de una proyección contralateral desde núcleo de la columna dorsal que concluía en el tálamo. Estudios más recientes de marcaje con cobalto en *Rana esculenta* (Antal y cols., 1980; Urbán y Székely, 1982), de trazado con HRP en *Xenopus laevis* (Nikundiwe y cols., 1982), o electrofisiológicos (Fernández de Molina. y cols., 1966; Urbán y Székely, 1982), han sugerido que el sistema columna dorsal-lemnisco medial en anfibios se asemeja al que existe en amniotas.

Mediante la utilización de técnicas electrofisiológicas, en experimentos con estimulación de la segunda raíz dorsal, del funículo dorsal o del núcleo de la columna dorsal, Urbán y Székely (1982) demostraron en *Rana esculenta* una proyección contralateral que alcanza el núcleo posterocentral talámico. Igualmente, los resultados procedentes de experimentos de trazado retrógrado han confirmado las proyecciones del lemnisco medial al tálamo en *Rana catesbeiana* (Neary y Wilczynski, 1977) y al torus semicircularis (Neary y Wilczynski, 1986; Neary, 1988).

En urodelos el núcleo de la columna dorsal *(núcleo funiculi dorsalis)* no ha sido descrito como entidad citoarquitectónica en el tronco cerebral (Herrick 1930, 1948; Kreht 1940a; Opdam y Nieuwenhuys, 1976), aunque, en experimentos de degeneración anterógrada, Nieuwenhuys y Cornelisz (1971) demostraron la presencia de una proyección organizada somatotópicamente, formada por las aferencias primarias espinales, que termina en la región caudal rombencefálica a nivel del óbex. Herrick (1948) postuló igualmente la terminación de fibras del funículo dorsal en dicha región, e indicó la posibilidad de que todas las fibras sensitivas somáticas que entran en el rombencéfalo, podrían terminar en un neuropilo común localizado a este nivel, en el que se combinan todas las modalidades sensoriales. Herrick (1948) sugirió que las fibras secundarias que se originan en dicha región rombencefálica no son estrictamente equipotenciales, y propuso una incipiente segregación de distintas funciones entre ellas. Del mismo modo, discutió la presencia del *"lemnisco bulbar general"* como un tracto mixto que asciende en la sustancia blanca ventrolateral del rombencéfalo, que recibe fibras contralaterales desde los núcleos del funículo dorsal y espinal del trigémino, y emite colaterales que alcanzan la formación reticular (Herrick, 1948; Herrick y Bishop, 1957). De acuerdo con estos trabajos, las fibras del lemnisco bulbar general se tuercen dorsalmente en la región del istmo y cruzan el mesencéfalo, en posición inmediatamente ventrolateral a las del lemnisco espinal, para terminar en el techo mesencefálico. Igualmente, postuló que algunas de ellas alcanzan la misma región talámica

que las del lemnisco espinal. Sin embargo, hasta el momento no existen apenas datos experimentales, disponibles en la bibliografía, basados en técnicas más modernas de trazado neuronal, sobre la presencia del lemnisco medial en urodelos, si bien, Wicht y Himstedt (1988) han descrito proyecciones rombencefalicotalámicas en *Triturus alpestris*, que alcanzan el tálamo dorsal.

La existencia del sistema columna dorsal-lemnisco medial, tanto en anuros como en urodelos, se ha descrito en base al patrón de terminación de las aferencias primarias espinales, así como en observaciones aisladas basadas en técnicas de trazado neuronal. En un estudio reciente con HRP sobre el desarrollo embrionario y larvario de las proyecciones ascendentes espinales en *Xenopus laevis* (ten Donkelaar y de Boer-van Huizen, 1991), se ha sugerido la existencia del sistema postináptico de la columna dorsal, si bien no se ha detallado, la localización de las neuronas que lo originan, el recorrido funicular de sus axones, ni su terminación en el DCN. Hasta el momento no se conoce con detalle la morfología o características de las células que dan origen al lemnisco medial ni sus principales destinos rombencefálicos, mesencefálicos y diencefálicos. Este hecho es consecuencia de la escasa diferenciación citoarquitectónica de los componentes de este sistema, que ha dificultado su estudio, así como por la falta de trabajos basados en técnicas de trazado e inmunohistoquímicas, que permitirian caracterizar su organización en los anfibios.

- Sistema espino-cervico-talámico y otras proyecciones incluídas en el funículo dorsolateral.

Hasta el momento no se ha descrito la existencia de los sistemas espino-cervico-talámico y espino-rombencefalico-talámico en vertebrados no mamíferos debido al escaso número de trabajos de investigación realizados sobre el tema. Sin embargo, existen algunos datos aislados en la bibliografía que apuntan la existencia de los tractos espinocervical y cervicotalámico, así como del núcleo cervical lateral (Herrick, 1948; van den Akker, 1970; Ebbesson, 1967; Finger, 1981; Forehand y Farel, 1982; Ito y cols., 1986; Necker, 1989; Ronan y Northcutt, 1990), con un modelo de organización similar al descrito en mamíferos.

Igualmente se han estudiado, en diversos vertebrados no mamíferos, las proyecciones espinales que ascienden en el funículo dorsolateral hasta el cerebelo, centros rombencefálicos como el núcleo del tracto solitario, núcleo del tracto descendente del trigémino, formación reticular lateral, y la región perifacial, y niveles de transición con el mesencéfalo como la región parabraquial (Pearson, 1936; Ebbesson, 1966, 1967, 1969; Karten, 1967; Jacobs, 1968; Pedersen 1973; Ebbesson y Hodde, 1981; Finger 1981; Funke y Necker, 1986; Funke, 1988), lo que sugiere una organización similar a la presente en mamíferos.

Funículo dorsolateral:

Funículos ventral y ventrolateral (cuadrante ventral):

- Sistema ventrolateral.

La disposición de proyecciones espinales que ascienden a través del cuadrante ventrolateral, para alcanzar diversas regiones rombencefálicas, mesencefálicas y el cerebelo, es un caracter común en el cerebro de todas las clases de vertebrados. Su presencia se ha descrito además de en los mamíferos (ver Willis y Coggeshall, 1991), en ciclóstomos (Tretjakoff, 1909; Pearson, 1936; Heier, 1948; Larsell, 1967; Northcutt y Ebbesson, 1980; Ronan y Northcutt, 1990), elasmobranquios (Ebbesson, 1972; Hayle, 1973; Ebbesson y Hodde, 1981; Smeets, 1982, Smmets y cols., 1983), teleósteos (Pearson, 1936; Hayle, 1973; Finger, 1978; Murakami y Ito, 1985; Ronan y Northcutt, 1990), anfibios (ver más adelante), reptiles (Huber y Grosby, 1926; Goldby y Robinson, 1967, 1969; Ebbesson, 1967; Pedersen, 1973; Northcutt y Pritz, 1978; ten Donkelaar y De Boer-van Huizen, 1978; Kusuma y cols., 1979; Ebbesson y Goodman, 1981; Künzle y Woodson, 1982; Pritz y Striztel, 1989) y aves (Karten, 1963; Karten y Rezvin, 1966; van den Akker, 1970; Vielvoye, 1977; Wild, 1983; Funke y Necker, 1986, Necker, 1989; 1990; Scheneider y Necker, 1989), en trabajos basados en técnicas degenerativas y de trazado neuronal.

La existencia de proyecciones espinotalámicas ha sido considerada como un caracter propio de vertebrados amniotas (ver Kevetter y Willis, 1984), debido a que su presencia fue únicamente descrita, además de en mamíferos, en el cerebro de aves (Karten, 1963; Karten y Rezvin, 1966, Wild, 1983; Necker, 1989; Scheneider y Necker, 1989, Funke, 1989b), y reptiles (Huber y Grosby, 1926; Ebbesson, 1967; Riss y cols., 1972, Kusuma, 1979; Pritz y Northcutt, 1980; Ebbesson y Goodman, 1981; Hoogland, 1981; Künzle y Woodson, 1982). En anamniotas, tanto en agnatos (Northcutt y Ebbesson, 1981, Ronan y Northcutt, 1990) como en elasmobranquios (Hayle, 1973) y teleósteos (Hayle, 1973), no se había descrito su existencia a excepción de en un trabajo en teleósteos, basado en técnicas anatómicas clásicas (Burr, 1928), y un estudio con técnicas de degeneración sobre el tiburón nodriza (Ebbesson y Hodde, 1981). Sin embargo, mediante trazado con HRP se ha confirmado la existencia de proyecciones espinotalámicas en teleósteos, que terminan mayoritariamente en el núcleo ventromedial (Murakami e Ito, 1985; Ito y cols., 1986). En anfibios, aunque su presencia fue sugerida por Herrick (1939), hasta el momento no existen evidencias que confirmen dicha proyección, si bien, no se han realizado hasta el momento experimentos con técnicas de trazado anterógrado.

En anfibios anuros (*Rana catesbeiana*) Ebbesson (1969, 1976) con técnicas de degeneración mediante hemisecciones espinales, describió que las fibras que ascienden a través del funículo lateral se agrupan fundamentalmente en dos tractos de fibras: un tracto medial, situado en los funículos ventral y ventrolateral que termina en niveles mesencefálicos; y un tracto lateral que se localiza en el funículo dorsolateral, en posición inmediatamente ventral al tracto descendente del trigémino. Éste último tracto asciende hasta el mesencéfalo, y en los casos en los que realizó lesiones inmediatemente caudales al óbex,

hasta el diencéfalo caudal. La mayoría de las proyecciones de ambos tractos terminan en la formación reticular del tronco cerebral, mayoritariamente en niveles caudales, de forma que el medial proyecta a los núcleos reticulares inferior y medio, mientras que el lateral alcanza el núcleo reticular superior, localizado en niveles más rostrales (Ebbesson, 1976). Además, a lo largo de su recorrido a través del tronco cerebral, el lemnisco espinal envía fibras al núcleo motor dorsal del vago, así como al núcleo del tracto solitario, núcleo motor del facial, el posible equivalente en anuros de la oliva inferior, el cerebelo, y de manera más dispersa a la sustancia gris periventricular en niveles del istmo. En el mesencéfalo, dicho autor únicamente describió una proyección poco desarrollada que termina en una zona descrita por Potter (1965a) como los núcleos laminar y magnocelular del torus semicircularis. Igualmente sugirió la existencia de una proyección espinotalámica directa, en aquellos experimentos en que las hemisecciones espinales fueron inmediatamente caudales al óbex, en los que el lemnisco medial podría estar implicado. Dicha proyección no fue observada en los casos con lesiones en el segundo segmento espinal o en niveles más caudales.

En anuros, únicamente existen estudios de las proyecciones ascendetes espinales basados en técnicas degenerativas (Ebbesson, 1969; Hayle, 1973a). La ausencia de proyecciones espinotalámicas directas en los citados trabajos, contrasta con las respuestas electrofisiológicas descritas en el tálamo, y basadas en experimentos con estimulación somatosensorial en el nervio ciático (Vesselkin y cols., 1971). Igualmente, resulta llamativo el patrón restringido de fibras espinomesencefálicas (Ebbesson, 1969; 1976) frente al mapa somatosensorial de representación de la superficie corporal presente en el torus semicircularis (Comer y Grobstein, 1981). La escasa diferenciación de las proyecciones espinotalámicas y espinomesencefálicas descritas puede ser debida a las limitaciones en la sensibilidad y resolución de las técnicas degenerativas. La utilización de técnicas de trazado retrógrado con HRP en experimentos con aplicaciones en el tálamo (Neary y Wilczynski, 1977) y en el torus semicircularis (Wilczynski, 1981, Wilczynski y Neary, 1986), no han permitido identificar la población neuronal que pudiera originar las proyecciones espinales ascendentes. Resulta necesario, por lo tanto, la utilización de técnicas más sensibles de trazado neuronal bidireccional con los nuevos y más sensibles trazadores Leucoaglutinina de *Phaseolus vulgaris* o dextrano aminas, con objeto de estudiar detalladamente las posibles homologías y diferencias existentes entre las proyecciones espinales ascendentes de anfibios y las de otros vertebrados.

En los urodelos *Ambystoma tigrinum* y *Ambystoma mexicanum*, Herrick (1914, 1939a, 1948) y Herrick y Bishop (1957) observaron un sistema que denominaron *lemnisco espinal*, compuesto por fibras de segundo orden que se originan en neuronas de la sustancia gris espinal y en segmentos inferiores rombencefálicos, y que ascienden hasta niveles cerebrales más rostrales. De acuerdo con la descripción de Herrick (1914, 1939a, 1948), dicho sistema asciende a lo largo del rombencéfalo, en posición inmediatamente ventral al tracto decendente del trigémino, asociado al tracto espinocerebelar, dando muchos fibras terminales en la formación reticular rombencefálica (Herrick 1914) y en el

cerebelo. En niveles más rostrales, la mayoría de las fibras terminan en el techo mesencefálico, aunque propone la posibilidad de que algunas de ellas continuen rostralmente para terminar en el tálamo dorsal (Herrick 1939b, 1948).

En un estudio posterior basado en técnicas de degeneración anterógrada en ajolotes juveniles, *(Ambystoma mexicanum)*, mediante hemisecciones en diversos niveles espinales, y seciones de raíces dorsales braquiales y lumbares, Nieuwenhuys y Cornelisz (1971) describieron un tracto que recibe fibras desde todos los niveles espinales, y asciende por la parte dorsal del funículo lateral. Este sistema, interpretado como el lemnisco espinal de Herrick, emite fibras en el rombencéfalo y en el cerebelo, terminando principalmente en el techo mesencefálico (Nieuwenhuys y Cornelisz, 1971), si bien la presencia de una proyección espinotalámica no pudo demostrarse. Los citados autores describieron el *sistema anterolateral* formado por fibras que se originan en su mayor parte en niveles cervicobraquiales, y ascienden, a través del funículo ventrolateral, hacia el tronco cerebral para terminar en la placa basal del rombencéfalo, aunque un pequeño componente alcanza el tegmento mesencefálico. Este sistema comprende además un conjunto de fibras que rodea a las del lemnisco espinal y termina dorsalmente en el área de transición entre cerebelo y el techo mesencefálico (Nieuwenhuys y Cornelisz, 1971).

Como en el caso de los anuros, en urodelos no se han realizado, hasta el momento, trabajos de trazado anterógrado sobre las proyecciones ascendentes espinales, y en el único estudio de trazado retrógrado efectuado con aplicaciones talámicas de HRP (Wicht y Himstedt, 1988) no se ha logrado confirmar las sugerencias de Herrick en cuanto a la existencia de la proyección espinotalámica.

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CAPÍTULO 2

Consideraciones técnicas 47

2.1.- *The use of in vitro preparations of the isolated amphibian central neuvous system in neuroanatomy and electrophysiology*

2.2.- Comentarios

The use of in vitro preparations of the isolated amphibian central nervous system in neuroanatomy and electrophysiology

2.1

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ABSTRACT

In the present study an isolated preparation of the complete anuran central nervous system (CNS) is described which can be kept alive for several days and allows tracing, immunohistochemical and electrophysiological studies. A simple perfusion chamber is being used in which the isolated CNS preparation is superfused with oxygenated Ringer. The use of an isolated CNS has many advantages including: 1) virtually all areas are easily accessible at the same time without having the problem of blood vessels that hinder access; 2) large lesions and massive tracer applications are possible without survival problems of the animal, and tracers will not be translocated by blood circulation; 3) since pulsations caused by the pressure changes of blood circulation do not occur, intracellular recordings are comparatively easy and stable, and 4) this approach offers the possibility to work on the same brain for

several days by storing the preparation in a refrigerator overnight at low temperatures, thus allowing extensive utilization of a single preparation and a reduction of experimental animals required. Some applications to the anuran auditory system illustrate that the isolated anuran CNS is well-suited for a variety of neuroanatomical and physiological techniques.

INTRODUCTION

Many isolated preparations of the central nervous system (CNS) have been developed and their success often depends on special features of the preparation that enable it to survive outside the body or on elaborate support strategies that reduce the effects due to absence of a blood supply. A factor that enhances viability of isolated CNS preparations is a decreased need for oxygen and metabolic substrates and this can be achieved in some cases by cooling mammalian preparations but is often found naturally in non-mammalian species.

Among such species are the lamprey and freshwater turtles. The lamprey spinal cord is thin, has no intrinsic blood vessels, and is oxygenated directly from the cerebrospinal fluid (CSF). The brain stem has intrinsic blood vessels, but is probably also oxygenated to a large extent from the CSF (Brodin and Grillner, 1990). Rovainen (1967a,b) took advantage of these favourable conditions by developing an *in vitro* preparation of the lamprey nervous system. The isolated spinal cord can be maintained *in vitro* for periods of 2-3 days at a temperature around 7-10¤C (Wallén et al., 1985). The brain stem is somewhat more sensitive but can often be maintained during a similar period of time (Brodin and Grillner, 1990). The turtle brain has an unusual resistance to anoxia (Lutz et al., 1985; Hounsgaard and Nicholson, 1990). *In vitro* preparations of isolated parts of the CNS, especially of the telencephalon (e.g., Connors and Kriegstein, 1986; Kriegstein and Connors, 1986; Larson-Prior et al., 1991) and of an isolated cerebellum-brain stem-spinal cord preparation (Keifer and Houk, 1989; Keifer et al., 1992; Sarrafizadeh and Houk, 1994), are used increasingly for combined tracing and electrophysiological studies as well as for pharmacological manipulations of the turtle brain.

For most mammalian tissues it is not possible to maintain adequate physiological integrity without perfusing the vascular system with some form of blood substitute, usually oxygenated artificial CSF. An example of such a preparation is the isolated guinea-pig brain (Llinás et al., 1981; Mühlethaler et al., 1993). Isolated neonatal CNS or brainstem-spinal cord preparations, however, can be kept alive without perfusion of the vascular system. An *in vitro* brainstem-spinal cord preparation is available for studies on the central control of respiration and locomotion (e.g., Smith and Feldman, 1987). An isolated CNS preparation of the newly born South American opossum, *Monodelphis domestica*, is a very attractive model for studies on the development and regeneration of synaptic interactions (e.g., Nicholls et al., 1990; Møllgård et al., 1994).

In vitro preparations of the amphibian CNS are increasingly used in physiological studies. Various approaches have been described including the use of a superfused preparation of *Xenopus laevis*

embryos (e.g., Kahn and Roberts, 1982; Roberts and Clarke, 1982; Roberts et al., 1986), brain slices (Holohean et al., 1990), brainstem preparations (Schmidt, 1976; Schaffer 1982; Cochran et al., 1987; Straka and Dieringer, 1993; Atzori and Nistri, 1994; Dicke and Roth, 1994; McLean et al., 1995), and combined spinal cord-muscle preparations (Sagawa et al., 1987; Wheatley and Stein, 1992). So far, the use of isolated CNS preparations for tracing experiments in anurans was restricted to a simple *in vitro* horseradish peroxidase (HRP) technique in which perfused and subsequently fixed brains are used (McCormick and Braford, 1984; González and Muñoz, 1987), and the cobalt-labeling procedure for fixed brains (e.g., Székely and Gallyas, 1975; Görcs et al., 1979). Straka and Dieringer (1991) used an isolated brainstem-spinal cord preparation for HRP tracing.

In the present study an isolated preparation of the complete anuran CNS will be described which can be kept alive for several days and allows tracing, immunohistochemical and electrophysiological studies. A simple perfusion chamber (modified after Schaffer, 1982) is being used in which the isolated CNS preparation is superfused with oxygenated Ringer. The use of an isolated CNS has many advantages. Some applications on the anuran auditory system will show that the isolated CNS is well-suited for a variety of neuroanatomical and physiological techniques.

MATERIAL AND METHODS

Various anuran species were tested for the applicability *in vitro: Rana temporaria, R. perezi, Bombina orientalis, Discoglossus pictus,* and *Xenopus laevis.* Animals were deeply anesthetized with tricaine methanesulphonate (MS 222) and cooled to a body temperature of 5¤C. The heart was exposed by rapid thoracotomy in order to perfuse the animal transcardially with approximately 40 ml iced Ringer solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose) that had been oxygenized with carbogen (95% O_2, 5% CO_2) to a pH of 7.3 (Straka and Dieringer, 1993). Head and vertebral column were then severed and the skin removed to avoid the spread of cutaneous toxins. The following preparation was carried out under a dissecting microscope in a dish the bottom of which was coated with Sylgard (Dow Corning) and covered with ice-cold Ringer solution. The skull was fixed with stainless steel needles sticked into the Sylgard layer. The complete brain and spinal cord were isolated by a dorsal or ventral approach by removing the overlaying bony tissue of the skull and the vertebrae. The nerves were cut with microscissors, special care was taken with the cranial nerves and especially the branchlets of the auditory nerve. After isolation which took approx. 10-15 min, the CNS was transferred into another dish with fresh iced Ringer. Subsequently, the pituitary gland, the dura mater, and the choroid plexus were removed to facilitate oxygen diffusion into the tissue. For ethical reasons, the telencephalon was removed (Mühlethaler et al., 1993). The application of tracer substances usually followed immediately. In case of intracellular studies, the CNS was isolated one day prior to electrophysiological recordings and was stored overnight at 6¤C in oxygenated Ringer solution.

Tracing techniques

Two different approaches, crystalline application and the injection of aqueous solutions, were used for the application of tracers. These were horseradish peroxidase (HRP, Sigma), biocytin (Ne-Biotinyl-L-lysine, Sigma), biotinylated dextran amines with molecular weights of 3,000 (3 kD BDA, D-7135) or 10,000 (10 kD BDA, D-1956), and various 10 kD or 3 kD lysine-fixable dextran amines conjugated to different fluorochromes, either to tetramethylrhodamine (RDA, D-1817 and D-3308, respectively) or fluorescein (FDA, D-1820 and D-3306, respectively). All dextran amines were purchased from Molecular Probes (Eugene, OR). For massive application of tracers, e.g., after hemisections or in case of superficial application sites, the substances were applied as small crystals dried onto the tips of glass microelectrodes or sharp tungsten needles. Since all substances tested are hydrophilic, the application area had to be rather dry to avoid undesirable spread of the dissolving tracer. A reduction of spread was achieved by taking the brain out of the Ringer solution for a short moment, and rapidly positioning the crystal with the aid of a micromanipulator. Immediately after the application, the brains were submerged again into the Ringer solution that was subsequently changed several times.

For the application of the dextran amine-coupled fluorescent dyes into deeper brain areas, we tested the pressure injection of aqueous solutions (approx. 5-10% substance in distilled water) via glass microelectrodes that were broken down to tip diameters of 10-30 µm and attached to a Hamilton syringe. The electrodes were then positioned with a micromanipulator and 1-10 µl of the solution were injected. With this approach, the injection speed turned out to be a crucial parameter since rapid injection (more than 1 µl per min) resulted in leakage of the substance along the penetration track.

Intracellular recordings of electrically evoked activity in the auditory pathway

During recording, the CNS was transferred into a perfusion chamber (modified after Schaffer, 1982) and fixed with stainless steel pins to the Sylgard covered floor of the chamber (Fig. 1). The chamber was mounted on an X-Y-table which allowed to move the CNS horizontally in relation to the recording electrode. The preparation was permanently superfused with freshly oxygenated Ringer solution. The flow rate was adjusted to 3-6 ml/min by a peristaltic pump (Masterflex Pump Controller). The solution was guided into the chamber by silicone tubing that ran through an ice container to maintain a superfusate temperature of 16¤ (Straka and Dieringer, 1993). Temperature and pH or oxygenation, respectively, were monitored continuously.

Single branchlets of the auditory nerve were stimulated electrically with suction electrodes. Stimuli consisted of monophasic square pulses (200 µs duration, 1-50 µA, 0.5 Hz repetition rate) and were delivered via an isolation unit (WPI, stimulus isolator A 360) which in turn was controlled by an interval generator (WPI, digipulser series 1800). Recordings were done with glass microelectrodes with an impedance of 80-120 MΩ when filled with 1-2 M potassium-acetate. After removal of the pia mater, electrodes were positioned stereotactically and lowered

into the brain with a piezo-driven system (Märzhäuser; PM 10-1). Recorded potentials were amplified (WPI, Cyto 720), digitized (Instrutech, VR100 A) and displayed on a personal computer screen for on-line control and stored on hard disk for off-line analysis.

For intracellular staining, electrodes were filled with a 3.5-4% solution of biocytin in 0.1-0.3 M potassium-acetate. Due to the low molarity of the solution, the impedance of the electrodes was much higher (up to 500 MΩ), but this did not influence the recordings significantly. Staining of a neuron was achieved by applying a constant current (1-3 nA) with the iontophoresis unit of the intracellular amplifier for 1-10 min. The membrane potential was controlled every minute to ensure the intracellular position of the electrode. After iontophoresis, the electrode was immediately retracted out of the brain.

(Immuno)histochemical procedures

After extracellular application of the tracers or finishing the electrophysiological recordings the CNS was stored in Ringer at room temperature, the solution was changed several times and the pH was monitored to ensure sufficient oxygenation. Over night, brains were put into freshly oxygenized Ringer (pH 7.3; approx. 300 ml) and the container sealed air-tight and kept at 6¤C to slow down metabolism and oxygen consumption of the tissue. After 12 hrs at this temperature, the pH of the Ringer solution typically increased to 7.6-7.8. With this day/night protocol, transport times were usually 16-18 hrs for the 3 kD BDA or fluorescent dextran amines, 20 hrs for biocytin, the 10 kD BDA and the fluorescent dextran amines, and 45 hrs for HRP.

The subsequent steps were dependent on the tracer applied. In the case of HRP and biocytin, brains were fixed with 4% paraformaldehyde and 1.25% glutaraldehyde in phosphate buffer (pH 7.4), or 4% paraformaldehyde in phosphate buffer (pH 7.4) for BDA. Brains were then embedded in gelatine, polyacrylamide or embedding medium (Reichert-Jung) for sectioning on a freezing microtome or in agar or polyacrylamide for sectioning on a vibratome. Section thickness was 20 µm if the sections were directly mounted onto slides after sectioning, and 40-50 m if the following steps were carried out with free-floating sections. For the localization of biocytin and BDA, endogenous peroxidases were blocked by incubation in 0.5% H_2O_2 in phosphate buffer for 15 min. Sections were then rinsed several times, washed with 0.5% Triton-X 100 for 10 min and subsequently incubated with streptavidin-coupled HRP (Amersham; dilution 1:100 in phosphate buffer) for 2 hrs. Like the tracer HRP, the streptavidin (biocytin)- or avidin-biotin (BDA)-coupled HRP was then visualized with the chromogen DAB following a modified protocol of Adams (1981); the peroxide was produced by a glucose-oxidase-reaction (Shu et al., 1988). After the DAB procedure, sections were lightly counterstained with neutral red, dehydrated and coverslipped.

In the case of the fluorescent dextran amines, brains were fixed with 4% paraformaldehyde and sectioned on a freezing microtome (40 µm) or on the vibratome (100 µm). If immunohistochemical localization of neurotransmitters was desired additionally, standard procedures with fluorescent

secondary antibodies were applied. Sections were then dried quickly to avoid fluorescence fading and coverslipped with anti-fading medium (Serva, Fluoromount) or a glycerin-gelatin medium.

RESULTS

Tracing techniques

The application of HRP showed the well-known characteristics of this tracer: a dense spot of non-incorporated enzyme at the application site and intense retrograde as well as anterograde labeling of neuronal structures (Fig. 2A). The enzyme was taken up by the soma, by terminal structures, and by damaged fibers of passage; transport speed was approximately 0.5 mm/h. In contrast, the application of biocytin yielded intensely stained neurons at the application site without a diffuse background, indicating that most of the tracer had been taken up by the cells (Fig. 2B). Transport speed was high (2 mm/h) and comparable to the situation *in vivo* (Luksch and Walkowiak, in preparation). The uptake of biocytin was not restricted to the soma, and resulted in anterograde as well as retrograde labeling. Anterograde labeling (Fig. 2C) was stronger compared to HRP, whereas retrograde labeling seemed to be less intense. However, even the retrograde label achieved with HRP did not stain entire neurons but comprised only primary and secondary dendrites. 10 kD BDA labeling was comparable to that described for biocytin. A much faster axonal transport was observed, however, for 3 kD BDA. With this fast dextran amine Golgi-like labeling of the entire neurons including secondary and tertiary dendrites was achieved (Fig. 2D).

Figure 3 shows an experiment in *Xenopus laevis* in which ascending projections to the torus semicircularis are demonstrated with 3 kD BDA. Labeled cells are found in acoustic and vestibular cell areas, in lateral line related structures, in the dorsal column nucleus, in a lateral cervical nucleus and in the spinal cord. Comparable data were obtained from the other species studied.

The use of the dextran amine-coupled fluorescent dyes (FDA, RDA) yielded best results when applied in crystalline form, both for 3 kD and 10 kD dextran amines, probably since the concentrations in the tissue achieved with the injection of aqueous solutions were too low to result in intense labeling of cells. The transport speed of the 10 kD fluorescent dextran amines was slightly faster for the rhodamine-coupled derivative (3 mm/h) than for the fluorescein-coupled derivative (2 mm/h). Both tracers were transported retrogradely as well as anterogradely, and led to an intense staining of somata and terminal structures up to a distance of 20 mm (Fig. 4A). Like for 3 kD BDA, a faster axonal transport was observed when using 3 kD fluorescent dextran amines.

In the experiments in which fluorescent dextran amine-tracers and immunohistochemical techniques for neuromodulator localization were combined, the protocols did not interfere with each other significantly. Figure 4B shows an example of retrogradely labeled neurons of the nucleus laminaris (torus semicircularis) traced with rhodamine-coupled dextran amin (red) and terminal structures containing

the neuromodulator leucine-enkephalin detected with a secondary antibody coupled to FITC (green).

Intracellular recordings

The electrical stimulation at the auditory branchlets of the statoacoustic nerve led to neuronal responses in several nuclei of the auditory brainstem. Neurons in other areas of the brain, e.g., in the tectum mesencephali, did not show responses, indicating that the electrical stimulation selectively excited the auditory pathway. The quality of the nerve preparation had a strong influence on the stimulation current that was necessary to excite auditory nuclei; stimulation current was usually 2-10 μA in good preparations but had to be increased up to 50 μA if the nerve had been bruised or pulled. Intracellular recordings were possible for up to four days after CNS isolation without a noticeable loss of activity, a decrease of membrane potential in penetrated neurons, or any sign of tissue degeneration in stained structures compared to structures stained *in vivo*. However, the stimulation current had to be increased probably due to the squeezing of the branchlets with the suction electrode. The longest recording time for one individual neuron was 4.5 h which exceeded that in comparable *in vivo* recordings by far.

Intracellular application of biocytin yielded intensely stained neurons (Fig. 5). The tracer was distributed homogeneously in soma, dendrites and axonal structures; no gradient was observable so that the recording site in the neuron could not be detected. Transport of the tracer within the cell was fast (approx. 2 mm/h) and comparable to the transport speed found after extracellular biocytin application. The quality of cell staining depended on the duration of the iontophoreses and the current applied but also on electrode characteristics; in some cases, neurons seemed to be completely stained after application of 1 nA for only 1 min. Occasionally, we observed ensembles of stained neurons (2-3) lying closely together. Such simultaneously stained neuronal ensembles were likely no artifacts due to accidental extracellular application of biocytin, since they were also observed when the resting membrane potential of the injected neuron was still high after the iontophoreses, indicating an intracellular position of the electrode. These simultaneously stained neurons usually showed similar dendritic and axonal patterns.

Immunohistochemical procedures

The survival time of the preparation, which was defined as the time between the dissection of the animal and the fixation of the isolated CNS, ranged between 16 hrs for the 3 kD dextran amine tracing and 4 days for intracellular biocytin studies. Even after the longest survival times, no sign of tissue degeneration was observed. Labeled neuronal structures had an inconspicuous appearence and could be traced over long distances (several centimeters) without any sign of inhomogeneous tracer distribution or membrane disruption indicating that the fixation by immersion was sufficiently fast to avoid tissue damage. In general, every result obtained in the *in vitro* preparation was comparable to results collected with an *in vivo* approach. In those experiments where immunohistochemistry for neuromodulator localization followed the transport phase of the tracer, the distribution of the immunolabeled terminals (e.g., leucine-enkephalin) was indistinguishable from the

pattern found in *in vivo* brains that had been fixed by perfusion of an anesthetized animal.

Each of the various procedures tested for the localization of HRP, biocytin or BDA gave comparable staining of labeled structures. However, since the diffusion of the streptavidin- or avidin-biotin-coupled HRP complexes is limited, the thickness of the sections should not exceed 70 µm when processed free-floating or 20 µm when processed already mounted onto slides. Staining of erythrocytes that sometimes remained after the perfusion disappeared completely after the blocking of endogeneous peroxidases with H_2O_2; this step was only possible for biocytin and BDA.

DISCUSSION

Methodological considerations

Various physiological studies using *in vitro* brain approaches in amphibians have been published during the last decades, comprising superfused CNS preparations of *Xenopus laevis* embryos (e.g., Kahn and Roberts, 1982; Roberts and Clarke, 1982; Roberts et al., 1986) brain slices (Holohean et al., 1990), brainstem preparations (Schmidt, 1976; Schaffer, 1982; Cochran et al., 1987; Straka and Dieringer, 1993; Atzori and Nistri, 1994; Dicke and Roth 1994; McLean et al., 1995) and combined spinal cord-musculature preparations (Sagawa et al., 1987; Wheatley and Stein, 1992). To our knowledge, the use of the complete isolated CNS for tracing experiments has only been reported for HRP (McCormick and Braford, 1984; González and Muñoz, 1987; Straka and Dieringer, 1991).

A crucial question for the evaluation of data collected in such a preparation is whether they are comparable to findings *in vivo*. We think that the transferability is supported by several arguments. First, no cellular degeneration of the brains was observed even after several days in the Ringer solution; intracellularly stained neurons as well as anterogradely and retrogradely labeled structures showed no differences to neuronal structures stained *in vivo* (Straka and Dieringer, 1991; Walkowiak and Luksch, 1994; Muñoz et al., 1995). The pattern of labeling in experiments such as the one shown in Fig. 3 is comparable to that obtained in *in vivo* experiments (e.g., Wilczynski, 1981; Will et al., 1985; Feng and Lin, 1991). Second, intracellularly recorded neurons had resting potentials of up to -90 mV even after three days in the Ringer solution, indicating a good physiological state of the brain. Third, our intracellular recordings showed that the electrical stimulation of the auditory nerve elicited reactions in all nuclei of the auditory pathway including the dorsal medullary nucleus, the superior olive, various structures in the tegmentum mesencephali, and the torus semicircularis, but not in other brain areas. Since afferents to the midbrain include at least two synapses (up to four), the essential neuronal circuits seem to be intact. Additionally, we did not find general differences when recording *in vitro* and *in vivo* (Luksch and Walkowiak, 1993). Fourth, the immunohistochemical data on neuromodulator localization showed no differences when compared with results yielded in another study (Luksch and Walkowiak, 1992) where the brains were fixated by perfusion of the anesthetized animal. This finding indicates that even after several days *in vitro* cellular

synthesis and transport systems are functioning properly. Taken together, we think that in amphibians, the *in vitro* approach maintains a physiological status of the brain, allowing anatomical as well as physiological studies.

Of the tracing substances applied extracellularly, HRP has been used for several decades and is well characterized (Mesulam, 1982). In combination with a heavy metal-intensification (Adams, 1981) and the tissue preservating glucose-oxidase-modification (Shu et al., 1988), anterogradely and retrogradely labeled structures are intensely stained. However, even if the retrograde transport exceeds the anterograde one, retrograde labeling of neurons comprises only the soma and the main dendrites. Biocytin has been introduced as an extracellular neuronal tracer only recently (King et al., 1989). The uptake of biocytin seems to rely on a specific, sodium- and ATP-dependent mechanism at the soma (King et al., 1989), and biocytin was therefore characterized as an anterograde tracer. The amount of retrograde transport is described contradictory in the literature (King et al., 1989; Diamond et al., 1991; Izzo, 1991; Kenan-Vaknin et al., 1992) and might depend on the density of terminal structures at the application site (Lapper and Bolam, 1991). In our experiments, retrogradely labeled structures were only weakly stained but comprised all known afferents. The main advantages of biocytin are the fast and strong anterograde transport, a comparatively weak background at the application site which allows the precise identification of the neurons labeled, the possibility to block endogenous peroxidases and the sensitivity and variability of the detection system which allows DAB-precipitation as well as fluorescent label. Biotinylated dextran amines have similar advantages. These tracers are transported retrogradely as well as anterogradely. The retrogradely labeled neurons have an excellent dendritic filling, whereas the tracer can be identified at long distances from the injection site. Particularly the fast 3 kD BDA is extremely useful in the *in vitro* preparation.

Fluorescent dyes coupled to dextran amines have been introduced as neuronal tracers several years ago (Glover et al., 1986; Fritzsch and Wilm, 1990; Nance and Burns, 1990). Small dextran amines with a molecular weight of 3,000 diffuse faster than the larger 10 kD ones (Popov and Poo, 1992; Tao and Nicholson, 1992; Fritzsch, 1993). In the *in vitro* approach, the substances have characteristics comparable to the *in vivo* situation, i.e. they are transported anterogradely and retrogradely with 2-3 mm/h and are taken up by intact as well as damaged cells (Glover et al., 1986). Similar findings have been reported in a mammalian brain slice preparation (Boulton et al., 1992). The main advantages of the fluorescent dextran amines are their fast and bidirectional transport, the uptake by all cellular compartments, the possibility to apply two or three differently coupled substances for easy double and triple labeling and the ease of localization. Moreover, double-labeled structures can be analyzed with confocal laser scanning microscopy in great detail. One disadvantage is the instability of some fluorescent dyes (e.g., FITC) to ethanol treatment, thus allowing only very rapid dehydration or coverslipping with water-soluble media. Another problem arises if the combination of fluorescent tracers with immunohistochemistry is desired since

glutaraldehyde, which may be necessary for the binding of the antibody to its epitope, can not be used because it causes autofluorescence.

The use of biocytin for the intracellular staining of neurons has several advantages: Very short application time is required for complete staining of a neuron, the substance is transported rapidly and distributes homogeneously in the cell and the axon, the tip diameter of the electrodes can be small since the molecule is small and does not tend to clog, and the detection system is very sensitive. The finding of simultaneously stained neuronal ensembles is probably due to coupling of these cells via gap junctions (Simpson et al., 1977) rather than unspecific application of biocytin. Similar effects have been reported by other groups working with biocytin (Kawaguchi et al., 1989; Kita and Armstrong, 1991; Wiggers and Roth, 1994; Schulte-Mattler and Luhmann, 1995). This hypothesis is supported by the fact that simultaneously stained neurons usually had comparable dendritic and axonal organization. However, since a comparable percentage of neuronal ensembles is found *in vivo*, this finding can not be attributed to the isolated brain preparation.

General considerations

In our experiments with the isolated brains of amphibians, we did not find any technical limitations concerning the application of tracing substances. We have not tested the application of tracers via iontophoretic injections into deep tissue areas. However, since neuronal circuits remain intact and can be activated across several synapses, the exact localization of nuclear boundaries by multi-unit recordings and the subsequent stereotactic tracer injection (e.g., HRP and biocytin) as described elsewhere (Luksch and Walkowiak, in preparation) should be possible. Moreover, we have not tested the combination of biocytin-application and immunohistochemistry with different DAB-protocols as described by Norgren and Lehman (1990) or Veenman et al. (1992) to yield different light-stable reaction products, which should be possible as well. In general, we think that every tracing technique developed *in vivo* may be applied in an isolated brain preparation as well.

The analysis of physiological parameters in an isolated brain has some obvious limitations, e.g., a stimulation of sensory systems with physiological stimuli is excluded. On the other hand, the stimulation of sensory systems with electrical stimuli leads to an excitation of complete sensory pathways and complex reaction patterns in individual neurons comparable to the findings in intact animals. We therefore think that the basic physiology of neurons can be investigated well *in vitro*. In some cases, the complete deafferentiation of the brain even may be an advantage of this preparation. *In vivo*, many physiological parameters of the animal and the environment can not be controlled entirely, e.g., variations due to experimental conditions such as immobilization stress, changing oxygen supply, or the influence of other sensory modalities. The 'constancy' of these parameters is guaranteed in the isolated brain, offering the possibility to analyze the response of single neurons and networks to a reliably constant stimulation and to manipulate the network via the application of neuropharmacological agents or via the stimulation of different afferents.

Besides the limitations for physiological work mentioned above, the use of an isolated CNS has many advantages. First, virtually all areas are easily accessible at the same time without having the problem of blood vessels that hinder access. Second, large lesions and massive tracer applications are possible without survival problems of the animal, and tracers will not be translocated by blood circulation. Third, as pulsations caused by the pressure changes of blood circulation do not occur, intracellular recordings are comparatively easy and stable. Additionally, since the meninges can be completely removed, the penetration of the electrode is facilitated and no limitations to the electrode shape exist. Fourth, this approach offers the possibility to work on the same brain for several days by storing the tissue in a refrigerator overnight at low temperatures, thus allowing extensive utilization of a single preparation and a reduction of experimental animals required. Taken together, we think that the isolated frog CNS is well suited for a variety of neuroanatomical standard procedures and, furthermore, may bridge the gap between isolated cellular physiology and the analysis of complex brain functions.

The present study shows that an isolated anuran CNS preparation has many advantages. Adequate physiological integrity can be maintained without perfusing the vascular system as is necessary in isolated mammalian brains (e.g., Llinás et al., 1981; Mühlethaler et al., 1993). Therefore, no elaborate support strategy is necessary to reduce the effects due to absence of a blood supply. Isolated CNS preparations of anuran brains can be used with equal success and ease as the lamprey (e.g., Brodin and Grillner, 1990) and turtle (e.g., Hounsgaard and Nicholson, 1990; Keifer et al., 1992) preparations.

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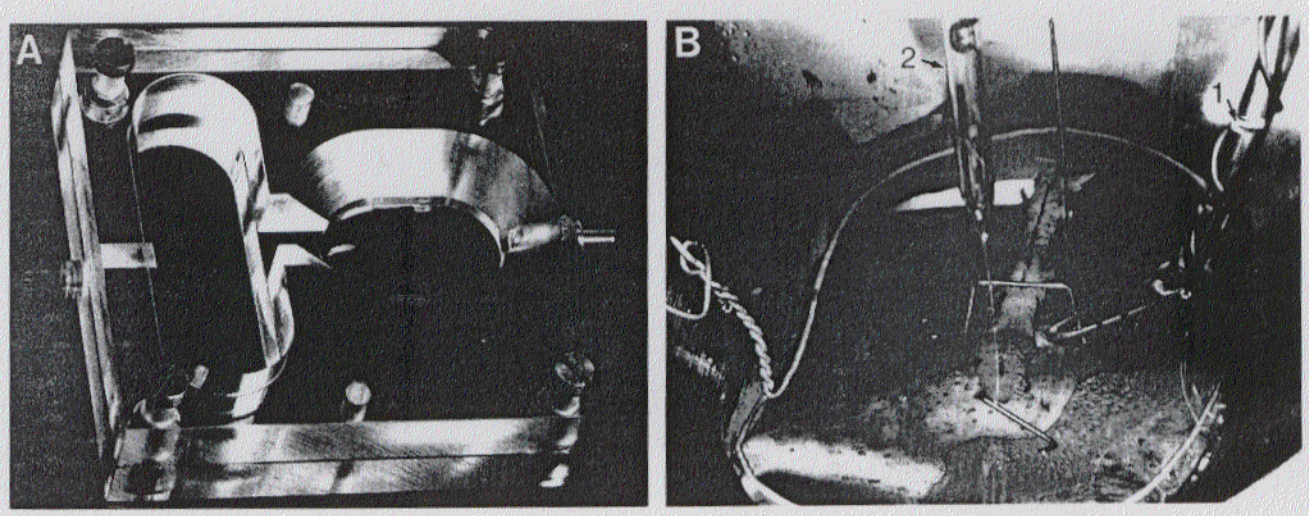

Figure 1: A, Recording chamber with Sylgard-covered bottom; B, Recording situation for electrophysiology: an isolated brain preparation of *Discoglossus pictus* is stimulated at a single branchlet of the auditory nerve via a suction electrode (1), the recording electrode (2) is lowered into the midbrain.

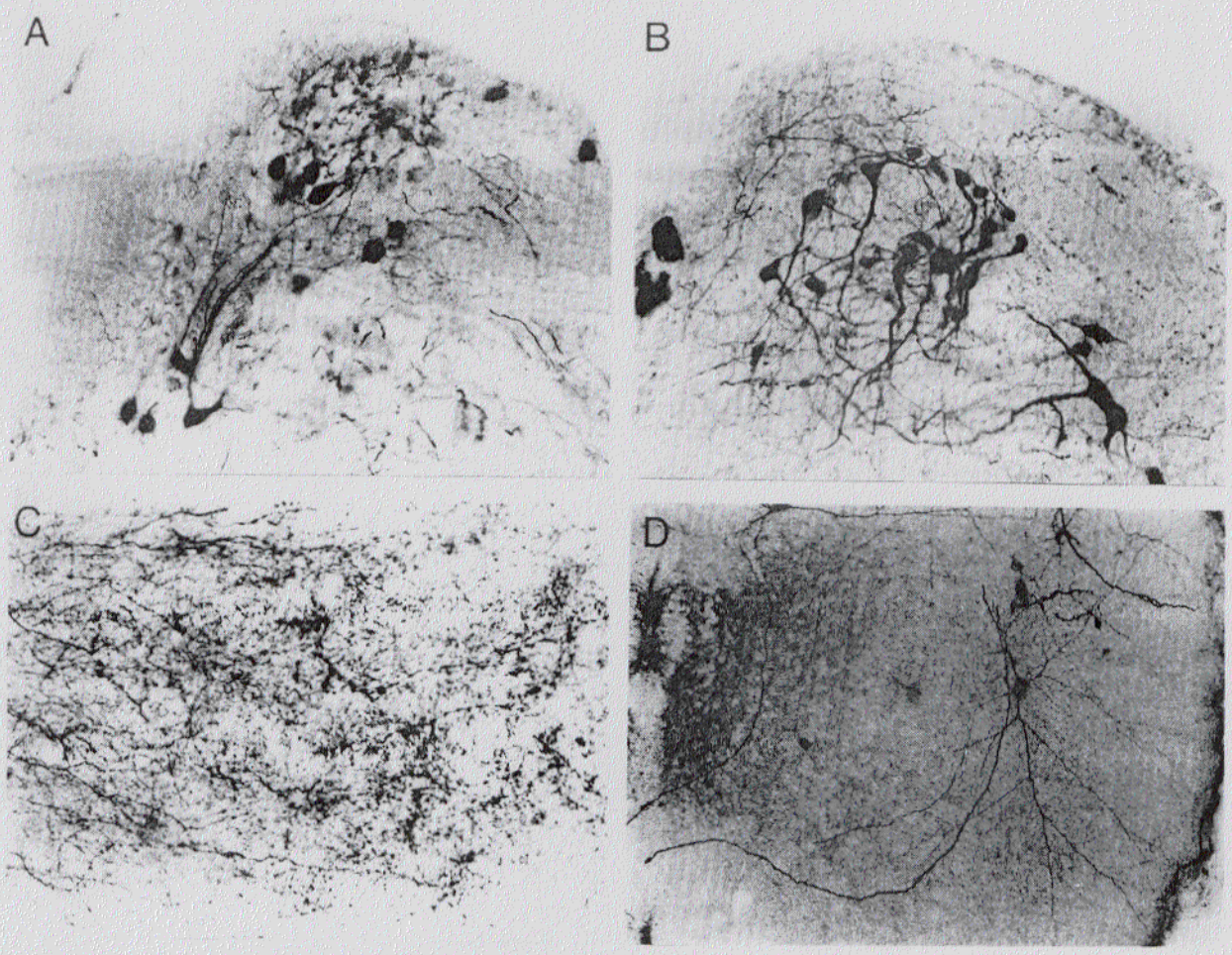

Figure 2: Examples of HRP, biocytin and BDA labeling in *in vitro* preparations. A, Retrogradely labeled neurons in the contralateral caudal and dorsal medullary nucleus as well as anterogradely labeled structures after application of HRP into the dorsal medullary nucleus of *Discoglossus pictus,* x175; B, Retrogradely labeled neurons in the contralateral dorsal medullary and vestibular nucleus after application of biocytin into the dorsal medullary area of *D. pictus,* x175; C, Anterogradely labeled fibers and terminal structures in the contralateral principal nucleus of the torus semicircularis after application of biocytin into the dorsal medullary area of *D. pictus*, x175; D, Retrogradely labeled neuron in the contralateral reticular formation of *Xenopus laevis* at the obex level after application of 3 kD BDA to the torus semicircularis, x190.

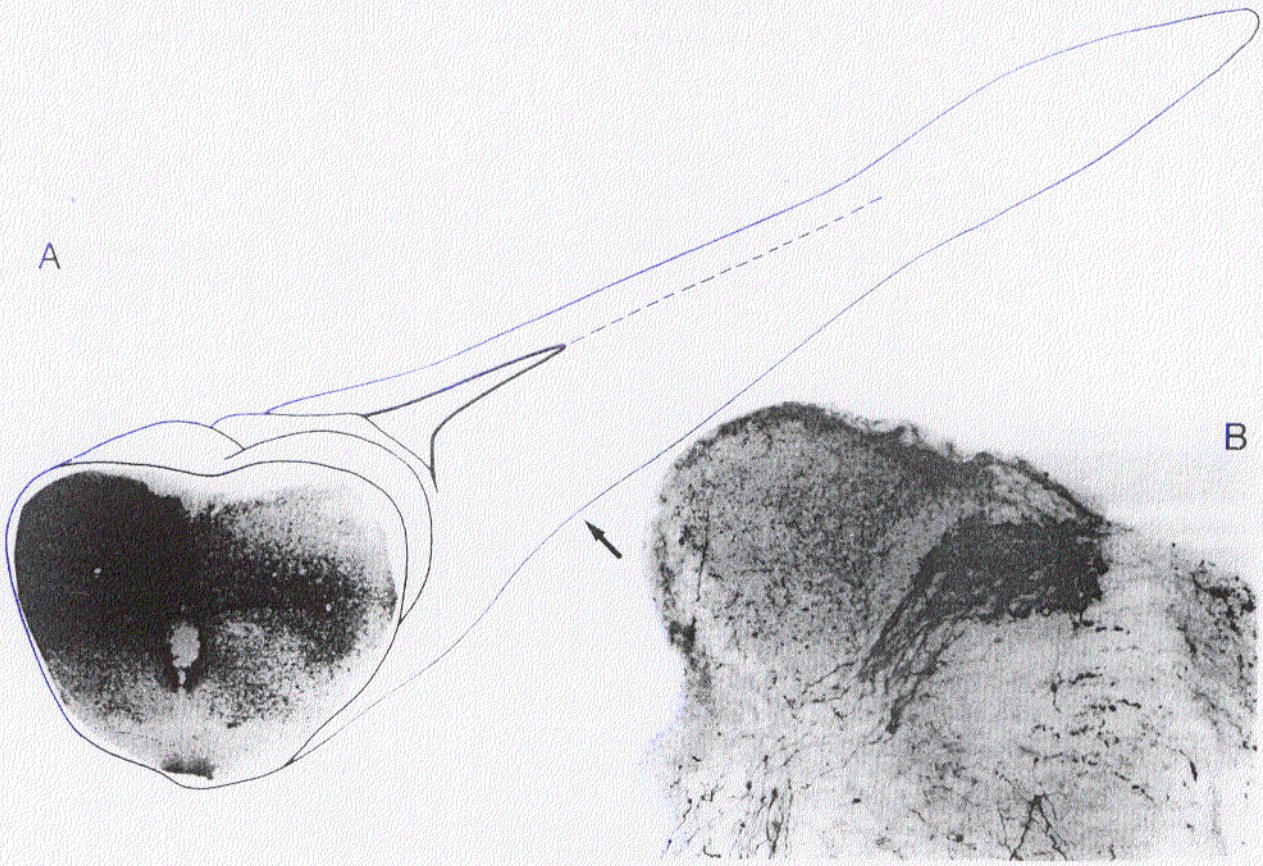

Figure 3: A, A representative experiment showing the application of 3 kD BDA to the torus semicircularis of *Xenopus laevis;* B, Example of labeling in the dorsal medullary nucleus, x170.

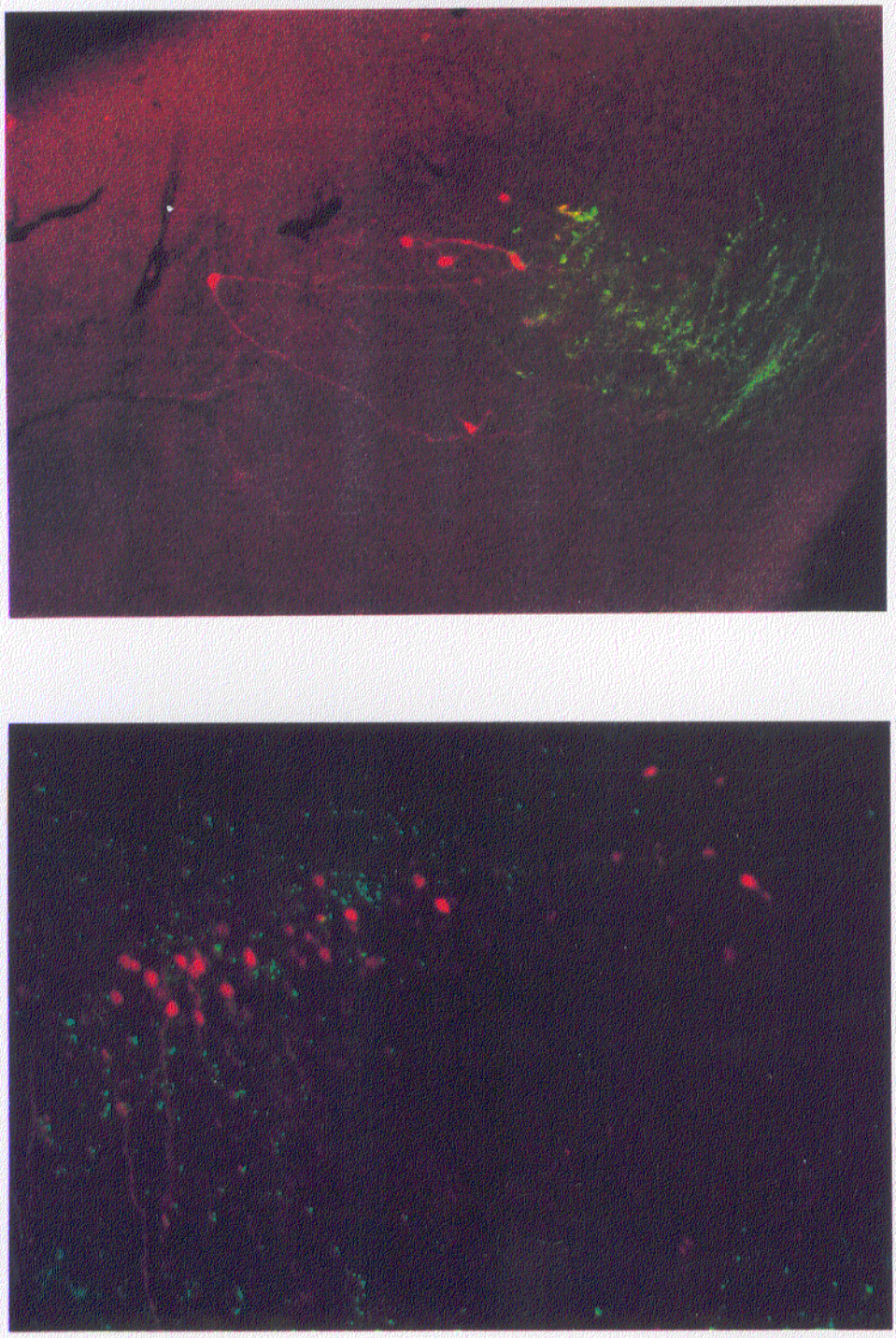

Figure 4: Double-labeled fluorescence preparations of *Discoglossus pictus*. A, Double labeling in the lateral part of the midbrain tegmentum. Red fluorescence: neurons of the lateral lemniscal nucleus retrogradely labeled with RDA from the contralateral torus; green fluorescence: fibers of the lateral lemniscus labeled with FDA from the ipsilateral torus, x250. B, Double labeling in the laminar nucleus of the torus semicircularis. Red fluorescence: neurons retrogradely labeled with RDA from the superior olive; green fluorescence: leucine-enkephalin-like immunoreactivity in the laminar nucleus, x250.

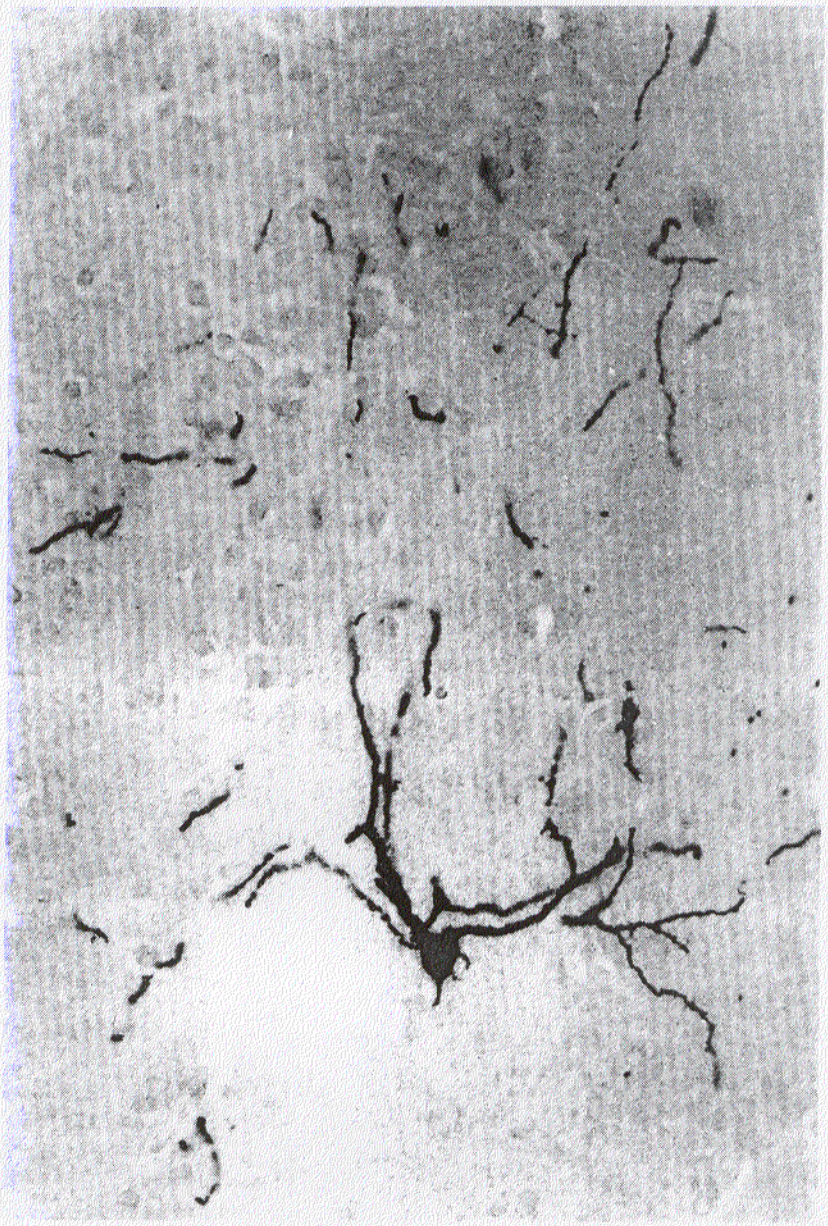

Figure 5: Intracellularly labeled neuron in the magnocellular nucleus of the torus semicircularis of *Discoglossus pictus*. Section thickness 20 µm, x225.

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The use of <u>in vitro</u> preparations of the isolated amphibian central nervous system in neuroanatomy and electrophysiology

COMENTARIOS

2.2

En el presente capítulo se describe, en diversas especies de anfibios, un protocolo para la realización de una preparación subperfundida con solución de Ringer oxigenada, en la que el sistema nervioso central (SNC) completo, y aislado del cuerpo del animal, puede mantenerse vivo durante varios días, permitiendo así la realización de estudios de trazado neuronal, con aplicaciones de trazadores tanto extracelular como intracelularmente; inmunohistoquímicos y electrofisiológicos.

La viabilidad de las preparaciones *in vitro* del SNC depende de aspectos como las estrategias de disminución de las necesidades metabólicas, así como el abastecimiento de oxígeno y sustratos metabólicos, que reducen los efectos causados por la ausencia del riego sanguíneo. En la mayoría de los tejidos de mamíferos no es posible mantener una integridad fisiológica adecuada, sin perfundir el sistema vascular con algún tipo de sustituto de la sangre. Un ejemplo de esto es la preparación del cerebro aislado de cobaya (Llinás y cols., 1981; Mühlethaler y cols., 1993). Sin embargo, las preparaciones aisladas del SNC neonatal de la zarigüella (Nicholls y cols., el 1990; Møllgård y cols., 1994), o preparaciones de tronco cerebral-médula espinal (Smith y Feldman, 1987), pueden mantenerse vivas sin perfusión del sistema vascular.

En vertebrados no mamíferos se han obtenido preparaciones *in vitro* viables para el estudio de partes del SNC, debido a características particulares. Así, en el caso del cerebro de la tortuga, al presentar una resistencia inusual a la anoxia (Lutz y cols., 1985; Hounsgaard y Nicholson, 1990), se han podido conseguir fácilmente preparaciones *in vitro* (Connors y Kriegstein, 1986; Kriegstein y Connors, 1986; Keifer y Houk, 1989; Larson-Prior y cols., 1991 Keifer y cols., 1992; Sarrafizadeh y Houk, 1994). En la lamprea la médula es delgada, carece de vasos sanguíneos intrínsecos, y es oxigenada directamente desde el fluído cerebroespinal; otras regiones, como el tronco cerebral, presentan vasos sanguíneos intrínsecos, pero probablemente son también oxigenadas en gran medida desde el fluído cerebroespinal (Brodin y Grillner, 1990). Todo ello favorece la realización de preparaciones *in vitro* viables (Rovainen, 1967a,b; Wallén y cols., 1985; Brodin y Grillner, 1990).

En anfibios se han descrito diversos protocolos para el estudio *in vitro* del SCN, incluyendo la utilización de una preparación subperfundida de embriones de *Xenopus laevis* (Kahn y Roberts, 1982; Roberts y Clarke, 1982; Roberts y cols., 1986), de secciones de cerebros (Holohean y cols., 1990), y de tronco cerebral (Schmidt, 1976; Schaffer 1982; Cochran y cols., 1987; Straka y Dieringer, 1993; Atzori y Nistri, 1994; Dicke y Roth, 1994; McLean y cols., 1995), así como preparaciones combinadas de médula espinal y musculatura (Sagawa y cols., 1987; Wheatley y Stein, 1992). Hasta el momento, el uso de preparaciones del SNC completo y aislado, para experimentos de trazado neuronal en anuros, se ha limitado a la utilización de la técnica de la peroxidasa de rábano (HRP) *in vitro*, en la que se emplean cerebros perfundidos y consecutivamente fijados (McCormick y Braford, 1984; González y Muñoz, 1987), y del método de marcaje con cobalto en cerebros fijados (Székely y Gallyas, 1975; Görcs y cols., 1979). Straka y Dieringer (1991) utilizaron una

preparación de tronco cerebral y médula espinal aislados para trazado neuronal con HRP.

En el presente trabajo se han empleado técnicas de trazado neuronal, mediante la aplicación extracelular de distintos trazadores en forma de cristal, y de inyecciones de soluciones acuosas. En general, las características observadas *in vitro* en cuanto a la captación del trazador, y a la direccionalidad y velocidad de su transporte son, en su mayor parte, comparables a las descritas mediante la aplicación *in vivo* de HRP (Adams, 1981; Mesulam, 1982), biocitina (King y cols., 1989; Diamond y cols., 1991; Izzo, 1991; Lapper y Bolam, 1991; Kenan-Vaknin y cols., 1992) y dextrano aminas con pesos moleculares de 3 kD o 10 kD, biotiniladas o conjugadas a diferentes fluorocromos, como tetrametilrodamina o fluoresceína (Glover y cols., 1986; Fritzsch y Wilm, 1990; Nance y Burns, 1990). Debido a que las dextrano aminas, de bajo peso molecular (3 kD), combinadas con biotina o con sustancias fluorescentes, difunden más rápidamente que las de alto peso molecular (10 kD) (Popov y Poo, 1992; Tao y Nicholson, 1992; Fritzsch, 1993), su utilización resulta sumamente útil en la preparación *in vitro*. Así, con la dextrano amina de 3kD combinada con biotina, se logró un rápido marcaje neuronal completo, incluyendo dendritas secundarias y terciarias.

Mediante la aplicación intracelular de trazadores *in vitro* se consiguen los mismos resultados que con las aplicaciones *in vivo*. Así con biocitina, ocasionalmente, observamos conjuntos de neuronas, simultáneamente marcadas, con patrones similares de morfologías dendríticas y axonales, probablemente debido al acoplamiento de dichas células por medio de uniones de tipo "gap" (Simpson y cols., 1977) a través de las cuales difunde la biocitina, más que a una aplicación inespecífica de ésta. Estos resultados, sin embargo, no pueden atribuirse a la preparación de cerebro aislado, debido a que se han descrito datos similares en estudios realizados *in vivo* (Kawaguchi y cols., 1989; Kita y Armstrong, 1991; Wiggers y Roth, 1994; Schulte-Mattler y Luhmann, 1995).

En los experimentos neuroanatómicos realizados en el presente trabajo, no hemos encontrado ninguna limitación técnica, en cuanto a la aplicación de los trazadores *in vitro*, ni signos de degeneración celular, incluso después de varios días en la solución de Ringer. Igualmente, no hemos observado diferencias morfológicas con respecto a los estudios realizados *in vivo*, en cuanto a las neuronas marcadas intracelularmente, las estructuras marcadas anterógrada y retrógradamente (Straka y Dieringer, 1991; Walkowiak y Luksch, 1994; Muñoz y cols., 1995), así como a su patrón de conectividad (Wilczynski, 1981; y cols., 1985; Feng y Lin, 1991).

Los resultados obtenidos en los experimentos inmunohistoquímicos, para la detección de Leu-Encefalina, y su combinación con técnicas de trazado con dextrano aminas *in vitro*, no mostraron diferencias, con los obtenidos en un estudio previo (Luksch y Walkowiak, 1992), en el que los cerebros fueron fijados, mediante la perfusión de los animales anestesiados. Esto indica que, incluso después de varios días en condiciones *in vitro*, los sistemas celulares de síntesis y transporte funcionan adecuadamente.

La estimulación eléctrica *in vitro* de las ramas auditivas del nervio estatoacústico condujo a la producción de respuestas selectivas en varios núcleos auditivos del tronco cerebral, incluyendo el núcleo rombencefálico dorsal, la oliva superior, diversas estructuras del tegmento mesencefálico, y el torus semicircularis; hasta cuatro días después del aislamiento del SNC, sin observarse una pérdida apreciable de actividad o una disminución del potencial de membrana de las neuronas penetradas, que mantuvieron potenciales de reposo de hasta -90 mV. El tiempo de registro más largo para una neurona individual fue de 4.5h, el cual excede considerablemente al conseguido en un registro comparable *in vivo*. Debido a que las aferencias al mesencéfalo incluyen por lo menos dos sinapsis (hasta cuatro), los circuitos neuronales esenciales parecen conservarse en esta preparación, la cual mantiene un buen estado fisiológico del cerebro. Además, en general no hemos observado diferencias entre los registros *in vitro* e *in vivo* (Luksch y Walkowiak, 1993).

El análisis de parámetros fisiológicos, en un cerebro aislado, tiene algunas limitaciones obvias, como la imposibilidad de excitar sistemas sensoriales mediante estímulos fisiológicos. Por otra parte, su excitación con estímulos eléctricos conduce a la estimulación de las vías sensitivas completas, y a patrones de reacciones complejas en neuronas individuales, comparables a los hallazgos en animales intactos. En algunos casos, la desaferentación completa del cerebro puede suponer una ventaja de esta preparación. *In vivo*, muchos parámetros fisiológicos del animal y el ambiente no pueden ser enteramente controlados, como por ejemplo, las variaciones debidas a las condiciones experimentales tales como el estrés causado por la inmovilización, el cambio en el abastecimiento del oxígeno, o la influencia de otras modalidades sensoriales. La preparación del cerebro aislado asegura la estabilidad de estos parámetros, ofreciendo la posibilidad de analizar la respuesta de neuronas únicas y de redes neuronales, ante una estimulación fehacientemente constante, y de manipulaciones por medio de la aplicación de agentes neurofarmacológicos o por la estimulación de distintas aferencias.

La utilización del SNC aislado ofrece numerosas ventajas para la realización de experimentos tanto anatómicos como electrofisiológicos. Todas las áreas cerebrales son fácilmente accesibles sin el inconveniente de la presencia de vasos sanguíneos que dificultan o impiden el acceso. En los experimentos anatómicos es posible realizar grandes lesiones y aplicaciones masivas de trazador, sin los problemas consecuentes de supervivencia del animal; además, los trazadores no son transportados por la circulación sanguínea. Los registros electrofisiológicos son comparativamente más fáciles de realizar y más estables que mediante la aproximación *in vivo*, al no producirse las pulsaciones ocasionadas por los cambios de presión de la circulación sanguínea. Igualmente, al ser posible extraer completamente las meninges, la penetración de los electrodos se facilita, y por tanto no existen limitaciones en cuanto a su forma. Esta aproximación experimental ofrece la posibilidad de trabajar sobre el mismo cerebro durante varios días, almacenando el tejido en un refrigerador durante la noche a temperaturas bajas, y permitiendo así su utilización

continuada, con la consiguiente reducción del número de animales de experimentación.

CAPÍTULO 3

Spinothalamic projections in amphibians as revealed with anterograde tracing techniques

3.1

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ABSTRACT

. Direct spinothalamic pathways were demonstrated in anurans (*Rana ridibunda, Xenopus laevis*) and in the ribbed newt, *Pleurodeles waltl*. With the powerful anterograde tracers *Phaseolus vulgaris* leucoagglutinin and biotinylated dextran amine rather extensive spinothalamic projections were found, including the ventromedial thalamic nucleus, the dorsal thalamus and several posterior diencephalic nuclei (anurans), and the neuropil lateral to the pars ventralis thalami as well as to the anteroventral and posterodorsal zones (*P. waltl*), respectively.

The presence of spinothalamic pathways appears to be a shared character in the brain of amniotes. Thus, thalamic projections from second order neurons in the spinal cord have been extensively shown for mammals[25], birds[18], and reptiles[2,3,8]. Among anamniotes, a distinct spinothalamic projection has been demonstrated only in an advanced galeomorph shark, the nurse shark, *Ginglymostoma cirratum*[4], possibly a case of a non-homologous, independently evolved character[17].

In amphibians so far tracing studies failed to show spinothalamic projections, both in anurans[3,5,13] and in urodeles[14,23]. In the present study the presence (or lack) of spinothalamic projections in amphibians was studied with modern, more powerful, anterogradely transported tracers. Therefore, in the anurans *Rana ridibunda* and *Xenopus laevis*, and in the ribbed newt, *Pleurodeles waltl*, the tracers *Phaseolus vulgaris* leucoagglutinin (PHA-L) or biotinylated dextran amine (BDA) were selectively applied into the spinal cord, and the anterograde labeled fibers and terminals in the thalamus were examined. In addition, several experiments with spinal injections of horseradish peroxidase (HRP) were available for each species. Part of the results have been published in abstract form[10].

The data presented are based on a total of 18 adult specimens of *Rana ridibunda*, six of *Xenopus laevis* and 10 of *Pleurodeles waltl*. In addition several BDA experiments were done in late tadpole stages of *X. laevis*. The animals were commercially obtained (*R. ridibunda* and *X. laevis*) or captured in the wild (with permission from the Spanish Government) in the surroundings of Madrid (*P. waltl*). All experiments were carried out under surgical anaesthesia with tricaine methanesulphonate (MS 222, Sandoz). In three series of experiments, the tracers HRP (6 cases), PHA-L (11 cases), and BDA (12 cases) were injected unilaterally into the cervical, thoracic (three cases) or lumbar (three tadpoles) spinal cord. In addition, in five cases, BDA was applied to the spinal cord as crystals (recrystallized from a saturated solution of the tracer in distilled water).

All injections were made iontophoretically by applying 5-8 μA positive pulsed current (7s on/7s off) to the tracer solution (15% HRP, 2% PHA-L or 10% BDA) in a glass micropipette (outer tip diameter 20-30 μm) for a period of 15-30 min. The animals were allowed to survive for 6-10 days in the experiments with PHA-L or BDA, and for 15-20 days in the HRP experiments. They were then reanesthetized with an overdose of MS 222 and perfused transcardially with 0.1 M phosphate buffer (pH 7.4), followed by a fixative containing 1% paraformaldehyde and 2.5% glutaraldehyde (for PHA-L and BDA experiments) or 1% paraformaldehyde and 1.25% glutaraldehyde (for HRP experiments) in 0.1 phosphate buffer. The brain and spinal cord were removed and further fixed for one to four hours in the perfusion mixture. They were then immersed in a mixture of phosphate buffer and 30% sucrose solution for three to five hours at 4¤C, subsequently embedded in a solution of 15% gelatin with 30% sucrose added, and stored overnight in a 4% formaldehyde solution at room temperature. Frozen sections were cut at 40 μm thickness in the frontal plane on a freezing microtome. Histochemistry for HRP followed a heavy metal intensification of the diaminobenzidine (DAB)-based HRP reaction product. For the immunostaining

for PHA-L the indirect peroxidase- antiperoxidase (PAP) technique was used, for visualizing BDA, a Vectastain ABC Elite Kit (Vector Laboratories).

The data obtained in *Rana ridibunda* and *Xenopus laevis* were largely comparable and a common pattern was found for the spinothalamic projection from the cervical spinal cord, as depicted in Fig. 1 for *Rana ridibunda.* The nomenclature for the different thalamic neuronal groups follows that of Neary and Northcutt[12] for the bullfrog, *Rana catesbeiana.* Application of each of the tracers into the spinal cord at cervical levels always resulted in the labeling of ascending fiber systems, among which a small contingent of fibers is seen to arise in the dorsal gray of the spinal cord, passing ventrally to cross the midline ventral to the central canal at the same spinal levels or immediately rostral to their cells of origin. In the contralateral spinal cord the fibers turn rostrally and ascend as the spinal lemniscus in the ventrolateral funiculus. A few uncrossed fibers course rostrally in the ventral funiculus. Throughout the brainstem, most of the fibers of the spinal lemniscus terminate in rhombencephalic and mesencephalic centers. However, a small component of fibers proceeds rostrally in the ventrolateral aspect of the caudal diencephalon. Here, most fibers bend dorsomedially towards diverse targets. At caudal diencephalic levels the dorsomedial part of the nucleus of the posterior tubercle (TP) is heavily innervated by labeled fibers, some of these terminate here whereas some others continue toward the dorsal thalamus (Figs. 1D, E; 3A, B). In the ventral thalamus, the ventromedial nucleus receives the heaviest spinal projection as thin varicose fibers that cross almost all of its cell layers (Figs. 1A, B; 3C). Fibers that reach this nucleus pass through the dorsal and ventral parts of the ventrolateral thalamic nucleus where also varicose structures were found. A small contingent of fibers arborizes within the limits of the posterior entopeduncular nucleus. In all the nuclei, their caudal aspect is always the most densely innervated. Fewer and scattered labeled fibers reach the dorsal thalamus and innervate the central, posterior and, to a lesser extent, anterior nuclei. In addition, the posterodorsal and posteroventral lateral nuclei are also scarcely innervated although numerous passing fibers cross towards the medially located dorsal thalamic nuclei. In the experiments where the application of the tracer was located in the thoracic spinal cord, similar, although less intensive, labeling was found in the thalamus, where the ventromedial nucleus receives the heaviest innervation. In three *Xenopus laevis* tadpole stages (stages 54, 57) a few ascending fibers from the lumbar spinal cord were found to innervate the ventral thalamus.

In the ribbed newt, *Pleurodeles waltl,* HRP applications to the spinal cord failed to label ascending projections as far as the diencephalon[10]. However, after PHA-L injections and BDA applications into the cervical spinal cord, a consistent and conspicuous labeling in the thalamus was found (Fig. 2). The different thalamic areas are termed after Wicht and Himstedt[23] for *Triturus alpestris.* As for anurans, ascending spinal fibers, predominantly contralateral to the injection side, ascend in the ventrolateral funiculus, pass through the brainstem, and enter the mesodiencephalic transition area. Here, the fibers form a neuropil just lateral to the cells in the pars ventralis thalami (Figs. 2B-E; 3D) that can be considered as a rostral continuation of a profuse terminal zone in the area equivalent to the torus semicircularis in the dorsal

mesencephalic tegmentum. Rostrally, labeled fibers ascend up to the level of the habenular commissure (Fig. 2A). Varicose fibers distribute primarily to the internal zone of the white matter, avoiding the periventricular grey where almost all of the cells are located. Within the dorsal thalamus, a few scattered fibers terminate in the posterodorsal zone (Figs. 2D; 3E) and also in the anteroventral zone (Figs. 2C; 3F).

The present study demonstrates the presence of rather distinct, direct spinothalamic pathways in amphibians. The use of new and more powerful anterograde tracers has made it possible to identify even fine terminal fields and thin scattered fibers in the thalamus of the three species studied. Although in anterograde degeneration studies in amphibians, distinct dorsal and ventral ascending bundles were found in the ventrolateral spinal funiculus, the most rostrally located targets of ascending spinal pathways were found in the mesencephalic tectum in the axolotl[14] and in the midbrain tegmentum of anurans[3,5], respectively. The lack of degenerating fibers or terminals in thalamic areas only confirmed other studies[5,17] in various anamniotes in which spinothalamic projections could not be observed. With the sole exception of an advanced galeomorph shark (the nurse shark, *Ginglymostoma cirratum*) in which spinal projections were found to reach the thalamus[4], it seemed likely that spinothalamic pathways evolved in amniotes only.

In anurans, however, electrophysiological studies[20] suggested a bilateral processing of somatic information all along the rostrocaudal extent of the dorsal thalamus. In the telencephalon several areas respond to somatic stimuli including the dorsal pallium, the medial pallium, the septum and the striatum[15,20]. All these telencephalic centers have been identified to receive thalamic information from the various nuclei that respond to somatic signals. Thus, the medial pallium, and the dorsal pallium are innervated by the anterior thalamic nucleus[7,11,16,21]. The striatum receives thalamic information from the central dorsal thalamus[22,24], and this thalamic region can be viewed as an intermediate in the transition of somatic information to the striatum. The present study shows that, apart from somatosensory information relayed to the thalamus via the dorsal column nucleus[9,13], a direct channel from the spinal cord is also available in anurans.

In urodeles, relatively few experimental data are available on ascending somatosensory pathways. In a pioneer study in *Ambystoma tigrinum*, Herrick[6] suggested a spinal innervation of the medial zone of the sensory dorsal thalamus. Anterograde degeneration[14] or HRP[23] studies could not confirm Herrick's ideas. In the present study in *Pleurodeles waltl* a small projection to the posterodorsal and anteroventral zones of the dorsal thalamus was shown. Both zones have been demonstrated to project to the striatum and the medial pallium, respectively[1,19,23]. Therefore, in urodeles as in anurans, various dorsal thalamic structures might serve as relay stations for somatosensory information from the spinal cord to the telencephalon.

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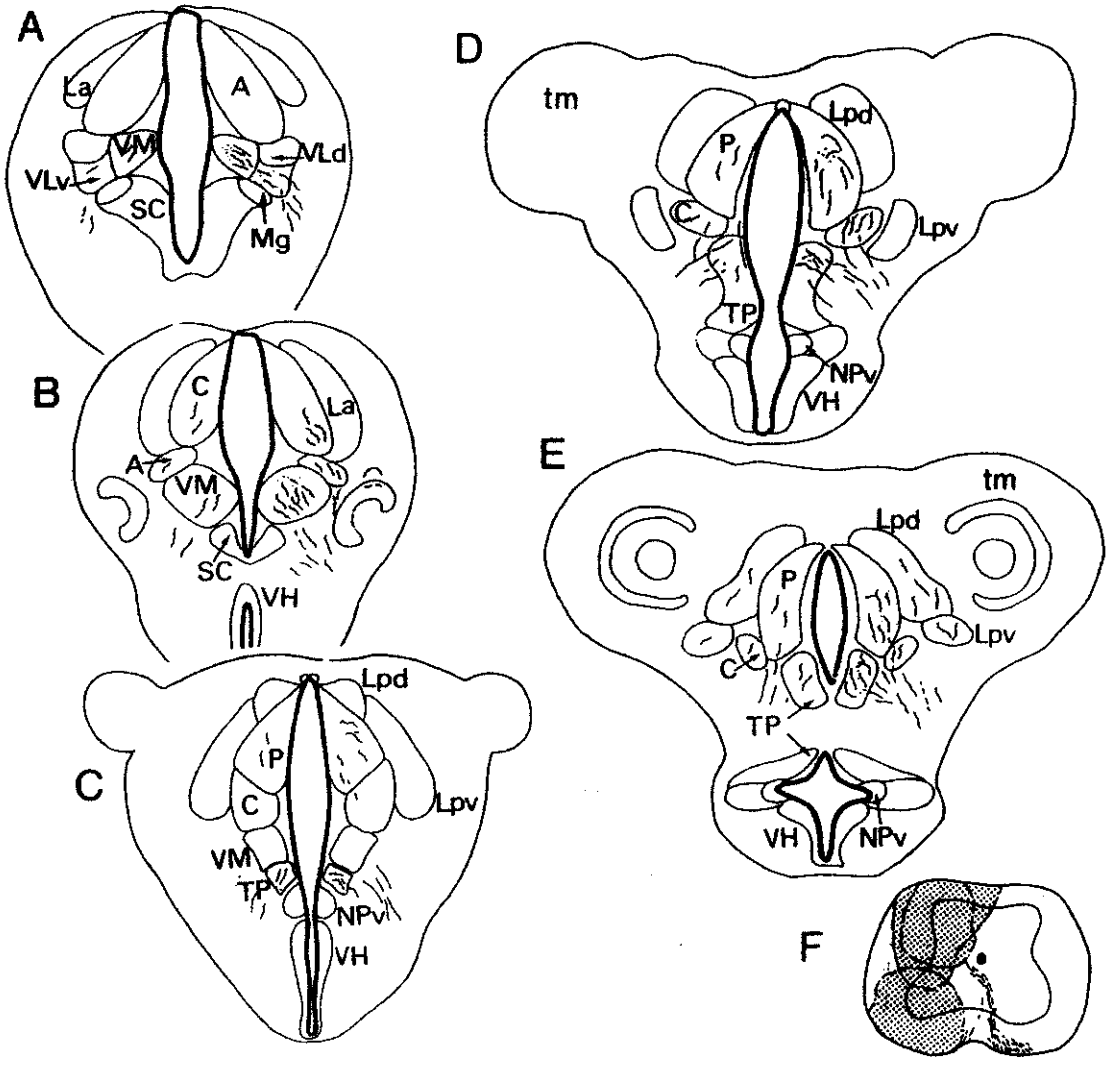

Figure 1: Camera lucida drawings of transverse sections through the diencephalon from rostral (A) to caudal (E) for a representative experiment with BDA application to the cervical spinal cord (shaded areas in F) of *Rana ridibunda*. Abbreviations: A, C, anterior and central thalamic nuclei; cho, chiasma opticum; La, Lpd, Lpv, lateral thalamic nucleus, anterior, posterodorsal and posteroventral divisions; Mg, magnocellular preoptic nucleus; NPv, nucleus of the periventricular organ; P, posterior thalamic nucleus; SC, suprachiasmatic nucleus; tm, tectum mesencephali; TP, nucleus of the posterior tuberculum; VH, ventral hypothalamic nucleus; Vld, Vlv, ventrolateral thalamic nucleus, dorsal and ventral parts; VM, ventromedial and thalamic nucleus.

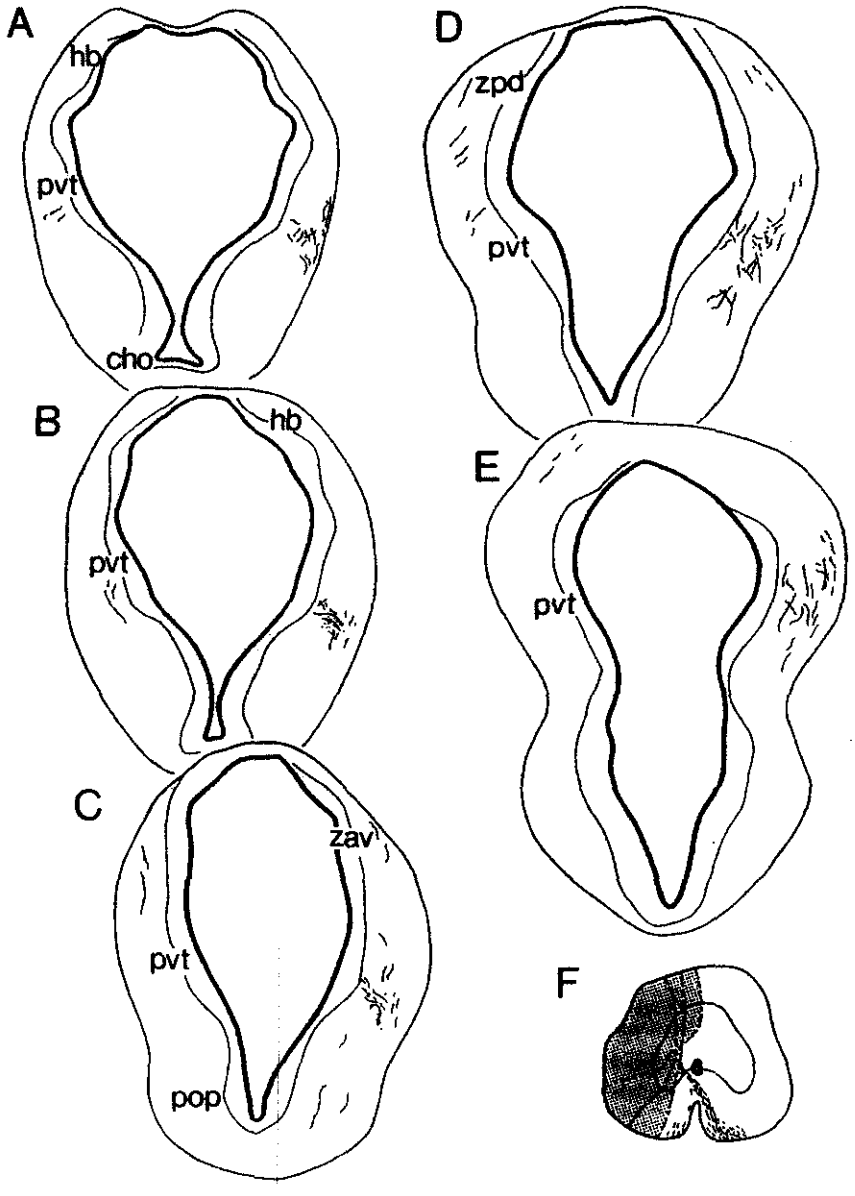

Figure 2: Diagrams of transverse sections through the diencephalon from rostral (A) to caudal (E) for a representative experiment with a PHA-L injection into the spinal cord of (shaded area in F). Abbreviations: cho, chiasma opticum; hb, habenula; pop, nucleus preopticus, pars posterior; pvt, pars ventralis thalami; zav, zona anteroventralis; zpd, zona posterodorsalis.

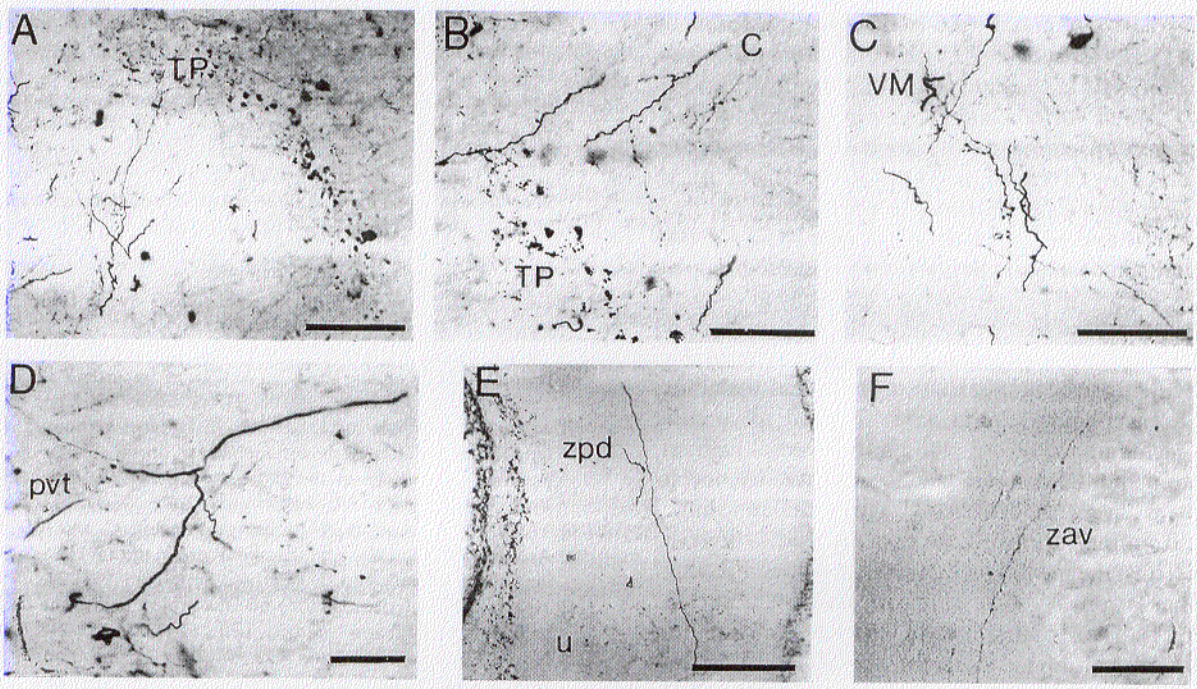

Figure 3: Photomicrographs of anterogradely BDA or PHA-L labeled fibers in *Rana ridibunda* (A-C) and *Pleurodeles waltl* (D-F). A: a bundle of fibers ascending toward the posterior tubercle; B, C, varicose fibers progressing dorsally to innervate the central (B) and ventromedial (C) thalamic nuclei; D: a dense arborization in the pars ventralis thalami contralateral side; E, F, a few, scattered fibers that reach the zona posterodorsalis or the zona anteroventralis in both the contralateral (E) and ipsilateral (F) side. Scale bars = 140 µm (A-C), 50 µm (D), 200 µm (E), and 100 µm (F). For abbreviations see Figs. 1 and 2.

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Spinal ascending pathways in amphibians: cells of origin and main targets

3.2

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ABSTRACT

As part of a research programme on the evolution of somatosensory systems in vertebrates, the various components of ascending spinal projections were studied with *in vivo* and *in vitro* tract-tracing techniques in representative species of amphibians (the large green frog, *Rana perezi*, the clawed toad, *Xenopus laevis*, and the ribbed newt *Pleurodeles waltl*). Three main ascending sensory channels were demonstrated:

1) Ascending projections via the *dorsal funiculus* include primary and non-primary projections that ascend to terminate mainly in the dorsal column nucleus at obex levels. A small component ascends farther rostralwards to terminate in the reticular formation, the octavolateral area, the trigeminal nuclear complex and in the granular layer of the cerebellum.

2) Projections ascending via the *dorsolateral funiculus* reach other spinal and supraspinal targets

than the dorsal funicular fibers, mainly ipsilaterally. At upper cervical cord and obex levels, many fibers innervate a region considered the amphibian homologue of the lateral cervical nucleus of mammals. In the medulla, these fibers ascend ventral to the descending trigeminal tract to terminate in the dorsal column and the solitary tract nuclei, and more rostrally, in the reticular formation, the descending trigeminal nucleus, and the medial aspect of the ventral octaval nucleus. Major projections reach the area between the facial motor nucleus and the ventral octaval nucleus, and a mediolateral subcerebellar band. These projections arise in neurons located mainly in the ipsilateral deep dorsal and lateral fields throughout the spinal cord.

3) Ascending spinal projections via the *ventral quadrant* of the spinal cord (the ventral and ventrolateral funiculi) ascend throughout the brainstem up to the diencephalon. Along its course this component innervates various parts of the reticular formation, the octavolateral area, the granular layer of the cerebellum, the region ventromedial and ventrolateral to the isthmic nucleus and the subcerebellar region. In the mesencephalon, the torus semicircularis, the midbrain tegmentum and, sparsely, the tectum mesencephali are innervated. Beyond the midbrain various dorsal and particularly ventral thalamic nuclei and the posterior tubercle are innervated by this ascending sensory channel. The cells of origin of some of these projections were observed in the dorsal, and to a lesser extent, in the lateral and ventral spinal fields of the spinal cord.

Evidence for the presence of these three main ascending sensory channels throughout vertebrates will be discussed. The presence of such channels appears to be a shared character in the brain of both amniotes and anamniotes.

INTRODUCTION

In terrestrial vertebrates, two basic systems of ascending spinal projections are found (see Willis and Coggeshall, 1991): 1) a *primary* afferent ascending spinal projection via the dorsal funiculus to the dorsal column nucleus, and 2) a *secondary* afferent projection via the lateral funiculus to the reticular formation, mesencephalon and thalamus. Recent studies in amphibians (A.Muñoz et al., 1994b, 1995 a,b) show that the dorsal column nucleus also receives non-primary spinal afferents, and that the dorsolateral funiculus innervates an anuran homologue of the mammalian lateral cervical nucleus. Both the dorsal column nucleus and the lateral cervical nucleus innervate the contralateral thalamus via the medial lemniscus. Moreover, distinct spinothalamic projections are present in amphibians (A. Muñoz et al., 1994a). These studies suggest that the classical subdivision of ascending spinal projections into two systems is too simple.

The present study will show that *three* main ascending sensory channels are present in vertebrates: an ipsilateral projection via the dorsal funiculus to the dorsal column nucleus, a mainly ipsilateral projection via the dorsolateral funiculus to a lateral cervical nucleus and various rhombencephalic centers, and mainly contralateral projections via the ventral and ventrolateral funiculi (the ventral quadrant of the spinal cord) to the brainstem and the thalamus. The ascending projections that course in the various funiculi of the spinal cord were studied with modern tract-tracing techniques in three representative species of amphibians: two anuran species, the Spanish large

green frog, *Rana perezi* (formely *R. ridibunda*), and the South African clawed toad, *Xenopus laevis*, and one urodele species, the ribbed newt, *Pleurodeles waltl*. The targets of ascending spinal fibers were studied with anterograde tracers (*Phaseolus vulgaris*-leucoagglutinin, horseradish peroxidase and biotinylated dextran amine). The cells of origin of some of these ascending sensory pathways were analyzed by retrograde labeling with HRP and BDA. The latter part of this study was done on an *in vitro* preparation of the anuran central nervous system, i.e. an isolated brain preparation (Luksch et al., 1996). It will be shown that the presence of three ascending sensory channels is common to vertebrates, and is a shared character in the brain of both amniotes and anamniotes.

MATERIALS AND TECHNIQUES

The present study is based on data obtained in the anuran species *Rana perezi* and *Xenopus laevis* and in the urodele *Pleurodeles waltl*. A total number of 20 adult *R. perezi*, 9 adult and 10 young adult *X. laevis* and 12 adult *P. waltl* were used. The animals were obtained from laboratory stock of the Department of Cell Biology, University Complutense of Madrid (*R. perezi* and *P. waltl*), and the Department of Animal Physiology, University of Nijmegen (*X. laevis*). *In vivo* and *in vitro* approaches were used.

In vivo tract-tracing experiments. All experiments were carried out under surgical anesthesia with MS 222 (Sigma). The anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (PHA-L, Sigma) as well as the bidirectionally transported tracers horseradish peroxidase (HRP, Boehringer), and biotinylated dextran amine (BDA 10kD, Molecular Probes) were applied to the dorsal horn of various spinal segments. Tracer solutions (a 10% HRP; a 2% PHA-L or a 10% BDA solution in 0.1M phosphate buffer-PB-pH 7.4) were iontophoretically injected during 5-10 min using a 5-10 μA positive pulled current (7 s on/7 s off) at cervical (brachial), thoracic or lumbar spinal cord levels in *R. perezi* and *X. laevis*. Cervical injections were made in *P. waltl*. In another set of experiments, BDA was recrystallized from distilled water onto fine sharp tungsten needles or glass micropipettes and applied dorsally at different spinal levels as well as in the torus semicircularis and the ventral thalamus of *Rana perezi* and *P. waltl*. Survival times varied from 5 to 10 days. The animals were then reanesthetized and perfused transcardially with isotonic saline followed by a fixative containing 4% paraformaldehyde for the PHA-L and BDA experiments, 1.5% paraformaldehyde and 2% glutaraldehyde for the HRP cases, in PB. The brain and spinal cord were removed, postfixed for four hours, cryoprotected in a 30% sucrose solution in PB, and embedded in gelatin or polyacrylamide (see ten Donkelaar and de Boer van Huizen, 1991). The brains were cut transversally at 40 μm on a freezing microtome. Histochemistry for HRP followed the heavy metal intensification of the diaminobenzidine (DAB)-based HRP reaction product according to Adams (1981). For visualizing BDA, an avidine biotin complex (Vectastain ABC Elite Kit, Vector Laboratories) was used. PHA-L was visualized with an indirect peroxidase anti-peroxidase (PAP) technique (Sternberg, 1979) using as antibodies: 1) goat anti-PHA-L (Vector) (1:2000) overnight at 4°C, 2) donkey anti-goat (Nordic) (1:50) for 1h at room temperature, and 3) PAP goat (Sigma) (1:800) for 1h at room temperature. The antibodies were diluted in 0.1% Triton X-100 in a 0.05M Tris/saline pH 7.6 buffer.

In some cases the sections were subsequently osmificated for 30-45 min in 0.1% osmium tetroxide in PB. Selected sections were counterstained with 0.1% cresyl violet solution. The sections were mounted on gelatin coated glasses and coverslipped with Entellan (gelatin embedded sections) or glycerin-gelatin (polyacrylamide embedded sections).

In vitro tract-tracing experiments. In 10 young adult *Xenopus laevis*, an *in vitro* approach was used according to Luksch et al. (1996) based on Cochran et al. (1987). The animals were deeply anesthetized with a 0.2% solution of MS222 and perfused transcardially with iced Ringer solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose; pH 7.4). The brains were removed, submerged in the same iced Ringer solution, and cut at middiencephalic or midmesencephalic levels. Applications of 3kD BDA (Molecular Probes, D-7135), recrystallized at the tip of sharp tungsten needles or glass micropipettes, were made with the help of a micromanipulator at the ventral thalamus, the torus semicircularis or at the dorsal horn of the spinal cord. The brains were kept for 18 hours at 15°C in continuously oxygenated Ringer solution (pH 7.4) with carbogen, and subsequently processed as described for the *in vivo* BDA experiments.

The nomenclature used in this study is largely based on studies by Ebbesson (1976) on the spinal cord, by Opdam and co-workers (Opdam and Nieuwenhuys, 1976; Opdam et al., 1976) and Nikundiwe and Nieuwenhuys (1983) on the brain stem, by Potter (1965) on the midbrain, by Neary and Northcutt (1983) and Wicht and Himstedt (1988) on the diencephalon, and by Northcutt and Kicliter (1980) on the telencephalon.

RESULTS

In the present study essentially two types of experiments were carried out. The supraspinal targets of ascending pathways from the spinal cord were studied with anterograde tracers. The cells of origin of spinoreticular, spinotoral and spinothalamic projections were studied with retrograde tracers. Some general remarks are appropriate here. Following iontophoretic or dry application of tracers, rather extensive ascending projections were found. The spinal cord gives rise to distinct, mainly ipsilateral, ascending projections via the dorsal funiculus and the dorsolateral funiculus and predominantly contralateral projections via the ventral funiculus and ventrolateral funiculus (the ventral quadrant of the spinal cord). Due to the proximity of the dorsal horn to the dorsal funiculus and the dorsolateral funiculus, tracer application to the dorsal horn often led to the involvement of the dorsal funiculus and the dorsolateral funiculus resulting in the uptake of tracers by its fibers. In line with previous studies (Antal et al., 1980; Nikundiwe et al., 1982; Jhaveri and Frank, 1983; ten Donkelaar and de Boer-van Huizen, 1991; A.Muñoz et al., 1995a) in such cases spinal dorsal root primary afferents were labeled as well as fibers of the postsynaptic dorsal column system. Moreover, in experiments with tracer applications at cervical and upper thoracic levels, cell groups such as the nucleus of the descending trigeminal tract or their descending fiber projections may have incorporated the tracer from the injection sites. In such cases, trigeminal primary afferents were labeled between the dorsal funiculus and the dorsolateral funiculus in line with previous data

(González and Muñoz, 1987; González et al., 1993). For the sake of clarity, anterograde tracing data will be presented for the dorsal and dorsolateral funiculi, and for the ventral quadrant, separately. In figures 1, 2 and 8 labeled fibers are indicated *only* for the dorsal funicular and the dorsolateral funicular components, whereas in figures 3 and 9 only the labeled fibers passing via the ventral and ventrolateral funiculi are shown. First, the anterograde tracing experiments in the anuran species studied will be discussed, followed by retrograde tracing data on the cells of origin of ascending spinal pathways, and finally the data obtained in *Pleurodeles waltl.*

Anterograde tracing experiments in anurans

In a first set of experiments, unilateral applications of the tracers PHA-L, HRP or BDA were made into the dorsal horn of the cervical spinal cord of *Rana perezi* and *Xenopus laevis*. The injections affected the dorsal and lateral spinal fields and, occasionally, the dorsal funiculus and the dorsolateral funiculus. More ventral injections affected the ventromedial, ventrolateral and lateral motor spinal fields, and the ventral and ventrolateral funiculi.

Ascending spinal projections passing via the dorsal and dorsolateral funiculi

In those experiments that affected the dorsal and lateral grey spinal fields, rostral to the injection site anterogradely labeled fibers were observed in the dorsal funiculus and the dorsolateral funiculus that innervate different supraspinal targets. Two experiments will be described. Following a lumbar BDA application (Fig. 1) labeled fibers could be traced via the ipsilateral dorsal and dorsolateral funiculi. Most of the labeled fibers ascending in the dorsolateral funiculus turn dorsomedially at upper cervical segments and at the level of the obex, and profusely innervate the neurons in the dorsolateral grey. At these levels, the labeled fibers that course in the dorsal funiculus massively innervate the medial portion of the dorsal column nucleus (DCN) and the caudal aspect of the nucleus of the solitary tract (Fig. 1 F-H). Only a few fibers terminate in the contralateral DCN. Just caudal to the obex a band-shaped area in the grey was found where terminal fibers originating in the dorsal funiculus and dorsolateral funiculus overlap (Fig. 1 F-H). This band borders an unlabeled zone in the dorsolateral margin of the obex region. The latter zone is known to be occupied by descending fibers of the trigeminal tract and the cells related to them (González et al., 1993). The labeled fibers in both funiculi could be traced into the rhombencephalon where they shift to more ventrolateral positions. The fibers from the dorsolateral funiculus terminate diffusely in the lateral reticular zone dorsal to the IXth and Xth motor nuclei (Fig. 1 B-D). Some fibers continue rostrally and innervate the subcerebellar region (Fig. 1A). Labeled fibers ascending via the dorsal funiculus could be traced to the dorsolateral aspect of the rhombencephalon where they innervate the lateral cells of the reticular formation, the area of the nucleus of the descending tract of the trigeminal nerve and the ventral region of the octavolateral area. Only a few fibers reach the level of the trigeminal nerve root and no dorsal funicular fibers were labeled in the cerebellum and the subcerebellar region.

Following tracer applications to the thoracic cord the pattern of labeling in the brain stem is essentially the same Fig. 4A). Two distinct sites of termination of ascending spinal DLF fibers should be

emphasized: a zone in the lateral reticular formation between the IXth and VIIth motor nuclei and more, rostrally, the subcerebellar region with a few fibers entering the caudal aspect of the granular cell layer of the cerebellar plate. A small fiber bundle continues rostrally to terminate in the posterodorsal tegmental nucleus of the mesencephalon. The innervation of the mesencephalic tegmentum is more abundant in *Rana perezi* than in *Xenopus laevis*. The fibers ascending from the thoracic cord via the dorsal funiculus innervate the nucleus of the solitary tract, the nucleus of the descending trigeminal tract and the octavolateral rhombencephalic area. In *X. laevis*, these fibers reach the zone of the lateral line nuclei and tracts while in *R. perezi*, in which no lateral line system is present in the adult, the innervation is restricted to the ventral nucleus of the VIIIth nerve. The rostralmost fibers in both species studied reach the subcerebellar region with some fibers entering the cerebellar granule cell layer.

After a cervical BDA application (Fig. 2) the innervation pattern is similar to that in lumbar and thoracic cases, although the amount of labeling in the lateral reticular formation between the IXth and the Xth motor nuclei (Figs. 2 F-H; 4 C,D) in the subcerebellar region and in the cerebellum (Figs. 2 A, B; 4 B) is much higher. The rostralmost fibers from the dorsolateral funiculus abundantly innervate the caudal aspect of the mesencephalic posterodorsal tegmental nucleus. Some fibers even decussate in the anterior medullary velum to its contralateral part.

Finally, it should be emphasized that, following unilateral application of tracers to lumbar, thoracic or cervical spinal cord, a small contralateral component of ascending fibers in the dorsolateral funiculus was always labeled. This may be due to spread of the tracer to the contralateral side or its uptake by dendrites of contralateral spinal neurons extending into the site of tracer application.

Ascending spinal projections passing via the ventral quadrant of the spinal cord

In those experiments that affected the dorsal, lateral or ventral spinal fields, a distinct, bilaterally ascending system from the spinal cord was labeled in the ventral quadrant of the white matter. One of these experiments is shown in figure 3, a cervical BDA application. It should be noted that in experiments with tracer applications restricted to the dorsal horn (dorsal and lateral spinal fields) more labeled fibers were found contralaterally than ipsilaterally in the ventral and ventrolateral funiculi. In cases in which tracer applications affected the ventral horn often the ventral and ventrolateral funiculi were damaged resulting in a higher amount of ipsilaterally than contralaterally labeled fibers. This is presumably due to labeling of damaged, crossed fibers. The axons of contralaterally projecting spinal cells could be traced from the injection site ventrally and medially, decussating to the contralateral side beneath the central canal, then turning rostralwards in the ventral and ventrolateral funiculi (Fig. 3V). Axons of ipsilaterally projecting cells were often seen to join the ipsilateral ventral funiculus. As the ventral quadrant component ascends in the rhombencephalon, it smoothly swings to more lateral and dorsolateral positions. Throughout the medulla it gives off thin varicose fibers to different targets (Fig. 3L-S). Most of the labeled fibers innervate structures in the caudal part of the brain stem. A progressive decrease in the amount of labeled fibers was observed as the ventral quadrant component

ascends to more rostral levels. Some of the thin terminal fibers that reach distinct medullary cell masses are collateral branches of thicker fibers located at the margin of the medulla that presumably ascend to more rostral levels. The inferior, middle and superior reticular nuclei receive an extensive innervation, from the ventral quadrant of the spinal cord, while the lateral reticular zone receives only a sparse innervation from this component. Additionally, the IX-Xth motor nuclei, raphe nuclei and the descending trigeminal tract are innervated. More rostrally, labeled fibers were observed in the area between the VIIth and Vth motor nuclei and more sparsely in the central grey at midrhombencephalic levels. A few, smooth fibers course dorsally into the octavolateral area to innervate the dorsal and ventral octaval nuclei (Fig. 3N, O). Some fibers enter the granular layer of the cerebellum where some of them cross the midline in the cerebellar commissure (Fig. 3J, K). A few fibers were observed in subcerebellar areas, just caudal to the isthmic nucleus. More rostrally, fine varicose fibers were observed ventromedial and ventrolateral to the conspicuous isthmic nucleus (Fig. 3 I) where the locus coeruleus and the nucleus of the lateral lemniscus are found. A sparse spinal innervation is also present in the band-shaped area located between the isthmic nucleus and the mesencephalic ventricle. The posterodorsal and posteroventral mesencephalic tegmental nuclei are sparsely innervated (Fig. 3G, H). At caudal mesencephalic levels ventral quadrant fibers turn dorsally along the lateral aspect of the midbrain and bend medially to terminate abundantly in the torus semicircularis (Figs. 3F-H; 4F). The principal, magnocellular and laminar toral nuclei receive spinal projections. A few fibers reach the midline where the commissural nucleus of the torus is located. Occasionally, some labeled fibers and terminals were observed at the lateral aspect of the mesencephalic tectum (Fig. 4E). In both species, at more rostral mesencephalic levels, the anterodorsal and anteroventral tegmental nuclei, the red nucleus and the interstitial nucleus of the fasciculus longitudinalis medialis are slightly innervated by spinal ventral quadrant fibers (Fig. 3E). In addition, scattered fibers distribute to the pretoral and pretectal grey and some fibers cross in the posterior commissure. Beyond the midbrain, both dorsal and ventral thalamic areas are innervated by ventral quadrant fibers (Fig. 3A-D). A few thin, varicose fibers innervate the posterior and central dorsal thalamic nuclei whereas the anterior nucleus receives only a sparse spinal innervation. In addition, the posterodorsal and posteroventral lateral nuclei are also sparsely innervated. The ventromedial thalamic nucleus and the dorsal aspect of the posterior tubercle are far more densely innervated. The fibers reaching all cell layers of the ventromedial nucleus pass through the dorsal part and, especially, the ventral part of the ventrolateral thalamic nucleus where varicosities were also found among its cells. No labeling was found more rostrally in the diencephalon or in the telencephalon in any of the cases studied.

After thoracic or lumbar HRP or BDA applications, a largely similar pattern of anterograde labeling, although less conspicuous, was observed. In all cases innervation of the aforementioned rhombencephalic and midbrain tegmental areas was observed. A low density of labeled fibers was present in the torus semicircularis and in the ventral thalamus. The latter structure was only very sparsely innervated in experiments with lumbar spinal injections.

Retrograde tracer experiments in anurans

BDA was used to trace the cells of origin of components of ascending spinal pathways. The tracer was applied to the ventral part of the thalamus, to the torus semicircularis and to the reticular formation. In a first set of experiments in *Rana perezi*, BDA was applied to the ventral part of the thalamus or to the torus semicircularis, two main targets of ascending sensory pathways from the spinal cord. After BDA application to the ventral thalamus, retrogradely labeled cells were observed, predominantly contralaterally, in the sensory trigeminal nuclei, in the dorsal column nucleus, in the lateral cervical nucleus and in the cervical, and to a lesser extent, thoracic spinal cord. Round, triangular and irregularly- shaped neurons were observed mainly in the dorsal spinal field and a few cells were present in the lateral field. Some piramidal and bipolar cells with dendrites extending predominantly horizontally were observed in the ventral fields of cervical segments. Higher numbers of retrogradely labeled cells were seen in the spinal cord in experiments with BDA applications to the torus semicircularis.

In a second set of experiments in *Xenopus laevis*, an *in vitro* approach was used. In an isolated brain preparation of young adult *X. laevis*, 3kD BDA was applied to the ventral part of the thalamus, to the torus semicircularis and to the reticular formation (Fig. 5). In such isolated brain preparations a more extensive pattern of labeling was observed. In all experiments labeled cells were observed more contralaterally than ipsilaterally. In experiments with ventral thalamic BDA applications (Fig. 5A) numerous labeled cells were present in the cervical spinal cord (Fig. 6A), whereas only a few cells were found at thoracic levels and no lumbar neurons were labeled at all. Most of the cells (80%) were found contralateral to the application site, and about 20% ipsilaterally projecting spinothalamic cells were observed. Cells with round, bipolar and irregular morphology were found.

After BDA applications to the torus semicircularis more retrogradely labeled cells were seen in the spinal cord (Fig. 5B). Moreover, labeled cells were observed throughout the spinal cord. Again, more contralaterally (65%) than ipsilaterally (35%) labeled neurons were found. This proportion remained constant throughout the rostrocaudal extent of the spinal cord. Most spinotoral cells (about 80%) were found in the cervical cord (Fig. 6C, D, F, G), about 10% at the thoracic level and about 10% in the lumbar cord. At cervical levels, labeled neurons were found predominantly in the ventral part of the dorsal horn. Most neurons have round or bipolar cell bodies with dendritic trees extending laterally towards the dorsolateral funiculus and within the dorsal field. Additionally, triangular and irregularly-shaped large cells with dendrites extending dorsally, ventrally and medially were present. The axons of these cells course ventromedially, cross the midline ventral to the central canal and join the contralateral ventral funiculus where they turn rostrally. Triangular or bipolar, horizontally oriented, ipsilaterally projecting cells send their axons to the ipsilateral ventral funiculus. Some neurons were also present bilaterally in the lateral field. Round and triangular as well as larger multipolar neurons were labeled bilaterally in the ventral spinal grey within the ventromedial, ventrolateral and lateral motor fields. At thoracic levels, neurons were also found particularly in the ventral part of the dorsal field. Small, round and large irregular cells were observed. The dendrites of the large, irregular neurons are directed dorsally and medially within the dorsal field and laterally towards

the dorsolateral funiculus. A few labeled neurons were present in the ventromedial and ventrolateral spinal fields. In some cases, the axons of ipsilaterally labeled cells follow an unusual course and cross the midline twice at the spinal level where the cell body is located, first dorsal and then ventral to the central canal to ascend in the ipsilateral ventral funiculus. In the lumbar cord, the dorsally located cell bodies, most of them contralateral to the application site, are bipolar and oriented horizontally, with medially and laterally directed dendritic processes.

Experiments with BDA applications to the rhombencephalic reticular formation were analyzed only in those cases in which the spread of the tracer into the adjacent white matter was minimal. After BDA applications to the reticular formation at the level of the inferior reticular nucleus (Fig. 5C), retrogradely labeled neurons were observed thoughout the spinal cord up to upper lumbar levels. Most cells were present in the cervical cord (Fig. 6B, E). In striking contrast to the thalamic and toral tracer application experiments, a more equal percentage of ipsilaterally (45%) and contralaterally (55%) projecting neurons was found. Their axons ascend at the ventral aspect of the lateral funiculus dorsal to those corresponding to spinotoral and spinothalamic projecting neurons. Medium-sized and large, fusiform and irregularly shaped neurons were found bilaterally in the deep dorsal field and, to a lesser extent in more superficial parts of the dorsal field. Their dendrites extend thoughout the dorsal horn and their axons are directed ventromedially or ventrolaterally to join the contralateral or ipsilateral ventrolateral funiculus, respectively. Additionally, a higher amount of ipsilateral than contralateral, round, irregular and fusiform medium-sized neurons were found in the lateral spinal field, with axonal processes directed to the ventrolateral funiculus. A few small, mainly round neurons were found, bilaterally, in the ventral horn. They were located in the ventromedial and ventrolateral fields.

Anterograde tracing experiments in an urodele

In the ribbed newt, *Pleurodeles waltl*, comparable experiments were carried out. Since hardly any data are available on dorsal root projections for urodeles, one experiment is included.

Ascending spinal projections passing via the dorsal and dorsolateral funiculi

In figure 7 an experiment is shown in wich a main brachial dorsal root was cut and subsequently labeled with BDA. In such experiments two fiber tracts were labeled in the spinal cord: a medial bundle in the dorsal funiculus consisting of thick fibers, and a lateral group of thin fibers within the dorsal portion of the dorsolateral funiculus, i.e. Lissauer's tract. Both fiber systems project to widespread spinal and supraspinal regions. Here only the distribution of dorsal root afferents to supraspinal targets will be discussed. Within the dorsal funiculus, spinal primary afferents ascend, somatotopically arranged, to the obex region. Fibers originating at cervical segments are present in the lateral part of the dorsal funiculus. In experiments in which a lumbar dorsal root was labeled, fibers were found in a position medial to those of cervical origin. These ascending spinal projections via the dorsal funiculus outline the DCN at the obex level (Fig. 7M, O). The terminal fields in this area largely resemble the organization of the fibers in the

dorsal funiculus. Thus, with a certain degree of overlap in the projection, medially situated axons from lumbar dorsal root ganglion cells terminate on medial cells in the DCN, whereas laterally located fibers arising from cervical dorsal root ganglion cells end on more lateral cells. Most of the primary afferents terminate dorsal to the cells of the DCN. However, some fibers reach more ventrolaterally located positions. Rostral to the obex level, tightly packed dorsal funicular fibers gradually turn ventrolaterally and ascend throughout the medulla dorsal to the descending trigeminal tract (Fig. 7J-H). Lumbar primary afferents could not be traced far beyond the rostral limit of the DCN. However, brachial primary afferents extend as far rostrally as the cerebellum where they arborize profusely within the granular layer (Fig. 7A, P). Throughout the rhombencephalon, varicose fibers leave the tract and arborize within the white matter where dendrites of the adjacent periventricular cells of the reticular formation, the nucleus of the solitary tract, the nucleus of the descending trigeminal tract and of the octavolateral area can be contacted. More rostrally, at the level of the VIIth and VIIIth nerve roots, some fibers enter the lateral reticular zone and the ventral aspect of the octavolateralis area.

After BDA applications to the cervical spinal cord in *Pleurodeles*, labeled fibers could be traced to supraspinal targets via the dorsal, dorsolateral, ventral and ventrolateral funiculi organized in a way similar to that in anurans, although some differences were eminent. One experiment is shown in figure 8. BDA was applied to the midcervical segment of the spinal cord (Fig. 8P). The application site included the dorsal and dorsolateral funiculi. In the dorsal funiculus, two different, adjacent components of labeled fibers could be distinguished (Fig. 8M-O): a medial component composed of thick, tightly packed fibers, and a second more dorsolaterally located component that is flanked laterally by the descending trigeminal tract. At spinal levels both components are very close to each other. At the obex level the medial component occupies a dorsomedial position and its thick fibers do not give off varicose terminal fibers. In turn the more sparse lateral fibers give off abundant thin, varicose fibers in this region that enter the dorsalmost aspect of the grey. Slightly rostral to the obex level, a small wedged-shaped, non-labeled area separates both components of labeled fibers (Fig. 8L, M). This is more evident rostrally in the rhombencephalon as the dorsal funicular system, the descending trigeminal tract and the dorsolateral funicular system swing ventrolaterally (Fig. 9A, B). A progressive decrease in the number of fibers of the medial component of the dorsal funicular fibers was observed up to the level of the facial motor nucleus where they fade and terminate as thick, varicose fibers in the lobe of the lateral line. However, the lateral component of the dorsal funicular fibers gives off thin terminal branches that massively reach the dorsal grey at caudal medullary levels. More rostrally, many fibers arborize within the white matter adjacent to the tract but some fibers reach the adjacent reticular, octavolateral and trigeminal areas as they ascend in the medulla up to the cerebellum. Here they distribute profusely to the ipsilateral superficial half of the granular cell layer (Fig. 8A).

Anterogradely labeled thin fibers in the dorsolateral funiculus ascend throughout the medulla just ventral to the descending trigeminal tract (Figs. 8, 9A, B). Along their course varicose terminal fibers were observed within the tract but they rarely enter the adjacent grey in the lateral reticular formaion, and the descending trigeminal nucleus. Numerous varicose

fibers and terminals were observed in the ventrolateral alar grey at the obex level and between the rostralmost root of the IXth-Xth complex and the root of the trigeminal nerve. Here some fibers turn toward the ventral aspect of the octavolateral area. More rostrally, at the level of the cerebellum, the dorsolateral funicular fibers shift dorsally and medially and profusely innervate the subcerebellar region. A few fibers enter the cerebellum. The rostralmost fibers sparsely innervate the caudal aspect of the mesencephalic tegmentum.

Ascending spinal projections passing via the ventral quadrant of the spinal cord

In experiments with BDA applications to the dorsal grey at cervical spinal segments a predominantly contralateral bundle of labeled fibers could be traced via the ventral and ventrolateral funiculi to the brain stem and diencephalon (Fig. 9). Most fibers cross the midline below the central canal and enter the contralateral ventral funiculus. Here, the fibers bend rostrally and ascend to various rhombencephalic, mesencephalic and diencephalic areas. Like in anurans, ascending spinal fibers passing via the ventral quadrant swing to a more lateral position in the rhombencephalon (Fig. 9F-L). In the rhombencephalon, projections were found throughout the rostrocaudal extent of the reticular formation (Figs. 9G-L; 10C), to the area octavolateralis and to the cerebellum (Fig. 9F). At caudal mesencephalic levels the ascending fibers bend dorsolaterally and innervate the outer layers of the tectum mesencephali (Figs. 9D, E; 10D), the torus semicircularis (Figs. 9D; 10E), and the midbrain tegmentum. Within these areas, the labeled fibers are mainly distributed in the inner region of the external white fiber layer, and hardly reach the periventricular cell layer. In the diencephalon, a main projection field is present in the ventral thalamus, and forms a rostral continuation of that observed in the torus semicircularis and dorsal midbrain tegmentum. Generally, in the thalamus, the fibers distribute in the outer fiber layer but do not penetrate the periventricular cell layer. A few fibers pass dorsal to the intermediate sulcus and innervate both the posterodorsal and anteroventral dorsal thalamic areas.

Retrograde tracer experiments

In experiments in which BDA crystals were applied to the ventral thalamic region or to the torus semicircularis of *Pleurodeles waltl*, retrogradely labeled neurons were found in the spinal cord. The majority of spinothalamic or spinotoral cells was observed in the dorsal grey of the contralateral cervical spinal cord (e.g., Fig. 10F). However, a small number of labeled cells was also present in the lateral and ventral fields of the grey. A small component of ipsilateral cells was also observed. In this set of experiments, no labeled cells were found more caudally in the spinal cord.

DISCUSSION

In the present study the mainly anterograde tracer PHA-L and the bidirectionally transported tracers HRP and 10kD BDA were used in an *in vivo* approach in adult *Rana perezi*, *Xenopus laevis* and *Pleurodeles waltl*. Additionally, in an *in vitro* approach 3kD BDA was used in isolated brain preparations of young adult *Xenopus laevis*. The results with both the *in vivo* and *in vitro* tracing techniques were largely comparable. However, the 3kD BDA *in vitro* approach presents

several advantages such as fast transport, the good neuronal labeling including secondary and tertiary dendrites, good labeling at long distances, the easy accessibility of all brain areas without the surgical and survival problems of the animals in cases with large lesion or massive tracer application and the precise tracer application under visual control (Luksch et al., 1996).

With these tracer techniques three main components of ascending spinal projections were demonstrated, i.e. a dorsal funicular component, a dorsolateral funicular component and a ventral quadrant component. In previous studies (A. Muñoz et al., 1994b, 1995a, b, 1996), spinal projections to the dorsal column and lateral cervical nuclei, respectively, were demonstrated. Additionally, the diencephalic targets of the ventral quadrant of the spinal cord were demonstrated (A. Muñoz et al.,1994a). In the present study the organization, the main targets and the cells of origin of some of the ascending spinal projections were investigated.

In previous studies, based on silver-stained material or making use of anterograde degeneration techniques, ascending spinal projections were demonstrated in anurans (Ebbesson,1969; 1976; Hayle, 1973a,b) and in urodeles (Herrick, 1914, 1930; Herrick and Bishop, 1958; Nieuwenhuys and Cornelisz, 1971). In *Necturus*, Herrick (1930) considered the ascending spinal projections through the dorsolateral funiculus as a distinct ascending spinal system, independent of the spinal lemniscus, and named it "the spinobulbar tract". At rhombencephalic levels it is located just ventral to the descending trigeminal tract and dorsal to the reticular neuropil. Additionally, Herrick (1930) observed a conspicuous terminal field of this spinobulbar tract just caudal to the obex and, rostrally, a close relationship of this ascending system with the reticular formation up to the level of the trigeminal nerve root. However, in later studies the ascending spinal projections via the dorsolateral funiculus were considered part of the spinal lemniscus (Herrick, 1948; Herrick and Bishop, 1958; Hayle, 1973a). In the axolotl, Nieuwenhuys and Cornelisz (1971) reported a distinct ascending spinal system in the dorsal part of the lateral funiculus that innervates the medulla and the mesencephalic tectum. It was interpreted part of Herrick's spinal lemniscus.

Additionally, Herrick (1930) described the spinal lemniscus as a system that ascends through the ventral quadrant of the spinal cord, separate from the spinobulbar tract at rhombencephalic levels by the reticular neuropil area. Subsequently, Ebbesson (1976) subdivided the ascending spinal projections in *Rana catesbeiana* that pass via the "anterolateral funiculus" into medial and lateral components. His lateral component is comparable to the dorsolateral funicular component, whereas his medial component forms part of our ventral quadrant component. However, Ebbesson's medial component predominantly innervates the inferior and middle reticular fields and fades at rostral rhombencephalic and caudal mesencephalic levels and his lateral component apperently innervates more rostral mesencephalic levels and even the thalamus, at least from the upper cervical cord. In the present study, based on more sensitive tract-tracing techniques, the dorsolateral funicular component was found to fade at cerebellar and caudal mesencephalic levels, whereas the fibers of the ventral quadrant component, located more ventrally throughout the rhombencephalon, turn dorsally at the isthmic level and reach mesencephalic and diencephalic targets (A.Muñoz et al., 1994a).

The spinal ascending pathways demonstrated in the present study will now be discussed in an evolutionary perspective.

Dorsal funicular pathways in amphibians

Tracer applications to the dorsal spinal cord at cervical, thoracic and lumbar levels showed that the fibers ascending via the dorsal funiculus and their pattern of termination in the DCN region are organized somatotopically, as previously noted in anurans (Antal et al., 1980; Nikundiwe et al., 1982; M.Muñoz et al., 1991; A.Muñoz et al., 1995b). The dorsal funicular fibers are ascending collaterals of primary afferents from spinal dorsal roots and most likely include second order projections towards the DCN, i.e. the postsynaptic dorsal column system (ten Donkelaar and de Boer van Huizen, 1991; A.Muñoz et al., 1995b). Part of the dorsal funicular component innervates rhombencephalic structures rostral to the DCN, in line with data obtained after labeling of spinal primary afferents (Joseph and Whitlock, 1968a; Antal et al., 1980; Nikundiwe et al., 1982; M.Muñoz et al., 1991; A.Muñoz et al., 1995b). The postsynaptic dorsal column system may contribute to this innervation. The most rostral site of termination of dorsal funicular fibers is the cerebellum. No dorsal funicular fibers from lumbar segments reach the cerebellum in line with previous data (Nikundiwe et al., 1982).

In urodeles, the glossopharyngeal and vagal nerves are known to send descending branches into the dorsal funiculus (Roth and Wake, 1985). Our BDA experiments on the ascending pathways of the cervical spinal cord of *Pleurodeles* (Fig. 8) showed two different components of labeled fibers in the dorsal funiculus. The lateral component is comparable to the dorsal funiculus of anurans and includes primary and, most likely, also non-primary ascending fibers. This component terminates mainly in the dorsal column nucleus at the obex level, but could be traced through the medulla up to the cerebellum, in line with our data on spinal primary afferents from the second spinal dorsal root (Fig. 7). Roth and Wake (1985) did not mention second dorsal root primary afferents reaching the cerebellum in lungless salamanders. The medial dorsal funicular component ascends, tightly packed up, to the area of the lateral line, where it fades, and corresponds to afferent fibers of the second root of the glossopharyngeal nerve as described by Roth and Wake (1985). Descending branches of this cranial nerve later mingle with spinal dorsal root projections in the dorsal funiculus up to the level of the fourth spinal nerve. Between the two dorsal funicular components an unlabeled region was found in *Pleurodeles* that may correspond to the area in which Fritzsch (1988) observed descending branches of inner ear afferents in various urodele species.

The dorsal funiculus is mainly aimed at the dorsal column nucleus at the spinomedullary border. This nucleus gives rise to a contralaterally ascending projection, i.e. the medial lemniscus, to the midbrain and thalamus (A. Muñoz et al., 1994b, 1995b).

Dorsal funicular pathways in vertebrates

Throughout vertebrates the dorsal funiculus contains ascending collaterals of primary spinal afferents reaching obex levels and continuing into the ipsilateral alar medulla (Table 1). In lampreys, dorsal funicular fibers innervate neurons at the obex level and in the octavolateral area (Northcutt and Ebbesson, 1980; Ronan and Northcutt, 1990). Dorsal column

fibers innervate the cerebellum as well (Ronan and Northcutt, 1990; Dubuc et al., 1993). In the Pacific hagfish, *Eptatretus stouti*, it is unclear whether dorsal column fibers reach the, little evident, cerebellum (Ronan and Northcutt, 1990). Although in the spotted dogfish, *Scyliorhinus canicula*, Hayle (1973a) was unable to trace degenerating fibers in the dorsal funiculus more than several segments rostral to spinal lesions, Ebbesson and Hodde (1981) demonstrated dorsal column fibers to a dorsal column nucleus at the obex level, and to the vestibular nuclear complex and cerebellar granule layer, in the nurse shark, *Ginglymostoma cirratum*. Experimental evidence for dorsal column pathways in bony fishes is limited. In the himé salmon, *Oncorhynchus nerka*, Oka et al. (1986) applied cobaltic lysine to the cut end of the spinal cord at the 10th to 15th spinal segment, and traced labeled axons via the dorsal funiculus to a site of termination at the obex level but hardly beyond the caudal medulla. Ronan and Northcutt (1990) reported the presence of dorsal funicular fibers to the obex and octavolateral area in African lungfishes (*Protopterus* species), bichirs (*Polypterus* species), and in the gar, *Lepisosteus osseus*. In bichirs and in gars, no dorsal funicular fibers were found to the cerebellum, but in lungfishes dorsal column fibers terminate in the granule layer of the cerebellum.

In amphibians, dorsal column fibers project to the ill-defined dorsal column nucleus, to the octavolateral area and to the cerebellum (Joseph and Whitlock, 1968a; Ebbesson, 1969, 1976; Hayle, 1973a.b; Antal et al., 1980; Székely et al., 1980; Nikundiwe et al., 1982; Urban and Székely, 1982; A. Muñoz et al., 1994b, 1995b, the present study; see Table 1). The dorsal column system has been extensively studied in reptiles. In the tegu lizard, *Tupinambis teguixin* (Ebbesson, 1967), a lacertid, *Lacerta viridis* (Jacobs, 1968), a boid snake, *Constrictor constrictor* (Ebbesson, 1969), a crocodilian, *Caiman crocodilus* (Ebbesson and Goodman, 1981), and in various turtle species, especially *Pseudemys scripta elegans* (Ebbesson, 1969; Pedersen, 1973; Künzle 1982; Künzle and Woodson, 1983) the dorsal funiculus innervates the dorsal column nucleus, vestibular nuclei, and the cerebellum. In *Thamnophis sirtalis*, Jacobs and Sis (1980) found a dorsal column projection to the DCN and to the descending vestibular nuclei. Comparable data were obtained in *Iguana iguana* (Joseph and Whitlock, 1968b) and in *Python reticulatus* (Kusuma and ten Donkelaar, 1980). In birds, Karten (1963) and van den Akker (1970) noted a spinal projection to the dorsal column nuclei of pigeons, *Columba livia*. In an HRP tracing study in pigeons, Wild (1985) found extensive dorsal root projections to the dorsal column nuclei, but also to a wide dorsolateral region of the rhombencephalon which includes the external cuneate nucleus, the lateral part of the nucleus of the descending trigeminal tract, and the principal trigeminal sensory nucleus. A discrete projection was also found to the nucleus of the solitary tract.

Ascending spinal fibers of both primary and non-primary origin are found in the dorsal funiculus of mammals (see Willis and Coggeshall, 1991 for review). The presence of non-primary spinal afferents to the dorsal column nucleus, i.e. the postsynaptic dorsal column system, has now been demonstrated throughout terrestrial vertebrates (e.g., Rustioni, 1973; Angaut-Petit, 1975a,b; Rustioni and Kaufman, 1977; Bennett et al., 1984; Giesler et al., 1984; Funke, 1988; ten Donkelaar and de Boer-van Huizen,

1991; Pritz and Stritzel, 1994; A. Muñoz et al., 1995b).

Dorsolateral funicular pathways in amphibians

In the present study a well-developed system of ascending fibers in the amphibian dorsolateral funiculus was demonstrated. The intraspinal targets of these fibers include the dorsal and lateral spinal field and, especially, the lateral cervical nucleus (A. Muñoz et al., 1995a, 1996). In the rhombencephalon, several structures are innervated by dorsolateral funicular fibers including the nucleus of the solitary tract, a lateral reticular zone and a subcerebellar "parabrachial" region.

In a previous *in vitro* tract-tracing study in *Xenopus laevis* (A. Muñoz et al., 1996), ascending spinal fibers from all levels of the spinal cord, passing via the dorsolateral finuculus, were found to terminate in a cell area ventrolateral to the dorsal column nucleus. This cell area can be considered a possible homologue of the mammalian lateral cervical nucleus. Moreover, this cell area was found to project contralaterally to the torus semicircularis and to the ventral thalamus, both targets for somatosensory projections. Similar observations were made in *Rana perezi* (A. Muñoz et al., 1995a). Data in *R. catesbeiana* tadpoles (Forehand and Farel, 1982) also indicate the presence of a lateral cervical nucleus in this ranid frog. The amphibian *lateral cervical nucleus* is innervated by the spinocervical tract which arises, mainly ipsilaterally, in the ventral part of the dorsal horn throughout the spinal cord (A. Muñoz et al., 1995a, 1996). In the present study three main other, rhombencephalic targets were found. In *Rana perezi* as well as in *X. laevis*, dorsolateral funicular fibers innervate the area of the nucleus of the solitary tract. Moreover, dendrites of the tyrosine hydroxylase-positive neurons, present in this nucleus, extend laterally and reach the dorsolateral funiculus (González and Smeets, 1994) where they may receive input from Lissauer's tract and from the dorsolateral funiculus. Spinal projections to the nucleus of the solitary tract were also observed in an anterograde degeneration study in *R. catesbeiana*. Hayle (1973a), however, did not report a spinosolitary projection in *R. temporaria*. In *Pleurodeles waltl*, the very poor segregation of neurons in the caudal part of the alar plate makes it difficult to distinguish which component may receive dorsolateral funicular afferents. In early studies in *Ambystoma tigrinum*, Herrick (1930) suggested that a spinobulbar tract, passing via the dorsolateral funiculus, contributes to a "general coordination field" at the obex level together with descending bulbospinal systems. The lateral reticular zone in the rhombencephalon is also innervated by dorsolateral funicular fibers. Especially, a lateral reticular area located dorsal to the VIIth-IX motor nuclei and ventral to the ventral nucleus of the VIIIth nerve, was labeled. In this area, retrogradely labeled cells were found after thoracic and cervical HRP injections (ten Donkelaar et al., 1981).

A distinct ipsilateral projection from the dorsolateral funiculus was found to a subcerebellar region. This projection reaches the caudal part of the secondary visceral nucleus as distinguished by Larsell (1923), Barnard (1936) and Opdam et al. (1976). Both Larsell (1923) and Barnard (1936) suggested a tractus visceralis secundarius connecting the nucleus of the solitary tract with the nucleus visceralis secundarius. This distinct nucleus lies ventrolateral to the nucleus cerebelli (Opdam et al., 1976). HRP injections

including the subcerebellar region resulted in labeling of cells in the nucleus of the solitary tract (González et al., 1984; A. Muñoz et al., 95b; in preparation). Tract-tracing studies in ranid frogs (Neary and Wilczynski, 1986; Neary, 1988, 1995) and in the green treefrog, *Hyla arborea* (Allison and Wilczynski, 1991), showed a distinct projection of a secondary isthmal nucleus in a position comparable to that of the nucleus visceralis secundarius to the ventral hypothalamus and to the preoptic area. Spinal projections were also found to arise in this "parabrachial" area (ten Donkelaar et al., 1981). In line with Neary's (1995) study, this subcerebellar region will be described as the *parabrachial nucleus*.

Dorsolateral funicular pathways in vertebrates

A spinocervical system, passing via the dorsolateral funiculus and terminating in a lateral cervical nucleus, appears much more common than previously thought (A. Muñoz et al., 1996). In anamniotes other than amphibians, evidence for a spinocervical tract projecting to a lateral cervical nucleus is at least suggestive. In petromyzontid and myxinoid agnathans (Northcutt and Ebbesson, 1980; Ronan and Northcutt, 1990) as well as in cartilaginous (Hayle, 1973a,b; Ebbesson and Hodde, 1981) and bony fishes (Hayle, 1973a,b; Finger, 1981; Ito et al., 1986) ascending spinal projections via the dorsal part of the lateral funiculus were demonstrated (Table 2). No separate site of termination, reminiscent of a lateral cervical nucleus, was noted. In reptiles, experimental evidence for the presence of a spinocervical tract comes from an anterograde degeneration study in the tegu lizard, *Tupinambis teguixin*. Ebbesson (1967) noted that at caudal brainstem levels some collateral fibers leave the dorsolateral funiculus and innervate an area located dorsal to the hypoglossal nucleus and ventral to the DCN. A tracing study in *Pseudemys scripta elegans* (Künzle and Woodson, 1982) also suggests the presence of a spinocervical tract. In birds, van den Akker (1970) showed a "dorsolateral ascending bundle" in the dorsolateral funiculus that arises in neurons found in the deep part of the dorsal horn. At cervical levels, this bundle innervates the deep dorsal and central spinal grey. More recent tract-tracing studies showed various ascending non-primary spinal projections in the dorsolateral funiculus of the pigeon (Funke and Necker, 1986; Funke, 1988; Necker, 1991), most likely including the spinocervical tract. The spinocervical tract in mammals has been studied extensively (see Willis and Coggeshall, 1991). It forms the first part of a bisynaptic spinocervicothalamic pathway (Morin, 1955; Nijensohn and Kerr, 1975; Boivie, 1983). Tract-tracing data in *Xenopus laevis* and in *Rana perezi* (A. Muñoz et al., 1995a, 1996) suggest the presence of an anuran homologue of the mammalian spinocervicothalamic system. Such a system is much more common in vertebrates than previously thought.

Spinosolitary projections passing via the dorsolateral funiculus were observed in reptiles (Ebbesson, 1967, 1969; Pedersen, 1973), in birds (Karten, 1963; Funke and Necker, 1986; Funke, 1988), and in mammals (e.g., Kuru, 1956; Hazlett et al., 1972; McMahon and Wall, 1983; Apkarian et al., 1985; Menétrey and Basbaum, 1987). Although in mammals the bulk of spinal projections to the reticular formation ascends via the anterolateral system (see Mehler, 1969; Willis and Coggeshall, 1991), ascending projections to different parts of the lateral reticular zone via the dorsolateral funiculus were

demonstrated (e.g., Zemlan et al., 1978; Blomqvist and Berkley, 1992). In cats, Blomqvist and Berkley (1992) showed spinoreticular fibers to the area surrounding the facial motor nucleus. They suggested that this termination field includes the feline adrenergic C_1 cell group in the rostral ventrolateral medulla caudal to the VIIth motor nucleus, and the catecholaminergic A5 group rostral to this motor nucleus. Neurons in the rostral ventrolateral medulla including the C_1 cell group project to the spinal cord via the dorsolateral funiculus (e.g., Ross et al., 1984; Dampney et al., 1987; Tucker et al., 1987). Although in the amphibian rhombencephalon the presence of adrenergic and noradrenergic cell structures in the rhombencephalon is not entirely clear (González and Smeets, 1994), comparison with the data available for mammals suggest than in amphibians the lateral reticular zone at the level of the VIIth motor nucleus is involved in viscerosensory functions.

The main rostral site of termination of ascending dorsolateral funicular fibers is the amphibian homologue of the mammalian parabrachial nucleus. In mammals, the parabrachial complex includes various subnuclei and the nucleus of Kölliker-Fuse (Saper, 1995). The parabrachial nucleus receives the majority of the ascending projections from the nucleus of the solitary tract (e.g., Herbert et al., 1990) as well as extensive projections from the spinal cord and different trigeminal nuclei (Bernard et al., 1995; Feil and Herbert, 1995). Several studies showed ascending spinal projections to the parabrachial nucleus via the dorsolateral funiculus (Nijensohn and Kerr, 1975; Björkeland and Boivie, 1984; Hylden et al., 1986; Kitamura et al., 1993; Slugg and Light, 1994; Bernard et al., 1995; Feil and Herbert, 1995). This bilateral projection arises mainly in laminae I-II of the dorsal horn (see Bernard et al., 1995; Feil and Herbert, 1995). This projection most likely plays a crucial role in a variety of important physiological systems, ranging from pain and automatic control through arousal and ingestive behaviour (Saper, 1995).

Some dorsolateral funicular fibers in amphibians continue rostrally and reach the caudal aspect of the posterodorsal nucleus of the mesencephalic tegmentum. This sparse projection may be reminisent of the spinomesencephalic projection via the dorsolateral funiculus in mammals to the periaqueductal grey and the nucleus cuneiformis (e.g., Nijensohn and Kerr, 1975; Zemlan et al., 1984; Björkeland and Boivie, 1984; Hylden et al., 1986; Yezierski, 1988; Bernard et al., 1995).

Ventral quadrant pathways in amphibians

In the anurans studied, in line with previous studies (Ebbesson, 1969, 1976; Hayle, 1973a), the bulk of fibers ascending via the ventral quadrant of the spinal cord fades at rhombencephalic levels. Ventral quadrant pathways terminate mainly in the reticular formation, particularly in its caudal, inferior part. Additionally, in the present study ventral quadrant projections were found to be lateral reticular zone, the raphe nucleus, the nucleus of the descending tract of the trigeminal nerve, the octavolateral area and the inferior olive. More rostrally, the parabrachial nucleus receives as sparse ventral quadrant projection, in addition to the massive innervation by the dorsolateral funiculus.

In the rostral part of the rhombencephalon, some fibers turn dorsally and enter the cerebellar granule layer. These fibers presumably arise from

neurons located mainly in the ventral horn of the spinal cord (González et al., 1984; Grover and Grüsser-Cornehls, 1984). This ventral spinocerebellar pathway forms one of the three spinocerebellar projections found in amphibians (Joseph and Whitlock, 1968a; Nieuwenhuys and Cornelisz, 1971; Hayle, 1973b; Ebbesson, 1976; Antal et al., 1980; González et al., 1984; Grover and Grüsser-Cornehls, 1984; the present study). The other two spinocerebellar projections in amphibians are the ipsilateral, primary spinocerebellar projection via the dorsal funiculus, and a non-primary dorsal spinocerebellar pathway via the dorsolateral funiculus that ascends through the medulla just ventral to the descending tract of the trigeminal nerve.

The ventral quadrant projection to the isthmic region found in the present study is in line with earlier anterograde degeneration studies (Ebbesson, 1969, 1976; Hayle, 1973a). In the mesencephalon, the torus semicircularis is the main target of ventral quadrant fibers. The torus semicircularis is a major integrating center for a number of sensory and nonsensory afferents in addition to auditory inputs, and may serve a role similar to the one the tectum mesencephali serves for the visual system (Wilczynski and Capranica, 1984). A distinct, mainly contralateral spinotoral projection arises from all levels of the anuran spinal cord. In *Rana catesbeiana*, Ebbesson (1976) noted a sparse spinomesencephalic projection to the magnocellular and laminar nuclei of the torus semicircularis. In the present study, a more extensive spinotoral innervation was found: spinal fibers mainly innervate the magnocellular and laminar nuclei but, although more sparsely, also the principal and commissural nuclei. More rostrally, labeled fibers were also found in the pretoral grey. Apart from this direct spinotoral input, the torus semicircularis receives somatosensory input from the nucleus of the descending tract of the trigeminal nerve (M. Muñoz et al., 1994), from the dorsal column nucleus (Wilczynski, 1981; Wilczynski and Neary, 1986; A. Muñoz et al., 1994b, 1995b) and from the lateral cervical nucleus (A. Muñoz et al., 1995, 1996). In *R. pipiens*, Comer and Grobstein (1981a,b) demonstrated the involvement of the torus in tactually elicited prey acquisition behavior. A crude somatotopic map of the contralateral body surface was found for the torus (Comer and Grobstein, 1981c). A sparse spinal innervation of the tectum mesencephalic was observed.

In the present study a direct, rather extensive, mainly contralateral spinothalamic projection was demonstrated. This projection arises mainly from cervical, but also from thoracic and, more sparsely, from lumbar spinal segments. Most cells of origin were observed in the contralateral dorsal horn. Spinothalamic fibers innervate the posterior tubercle, the ventromedial and the ventrolateral thalamic nuclei. Only a few spinal fibers reach the posterior, central and anterior thalamic nuclei.

In *Pleurodeles waltl*, the ventral quadrant pathways are comparable to those in anurans. A notable exception in the well-developed spinotectal component. The pattern of termination of spinoreticular projection is largely comparable to that found with the classical silver impregnation techniques (Herrick, 1948; Herrick and Bishop, 1958) and with anterograde degeneration techniques (Nieuwenhuys and Cornelisz, 1971; Ebbesson et al., 1972). The indistinct torus semicircularis, characterized by its octavolateral input (González and Muñoz, 1987), receives spinal input via the ventral quadrant pathways. In *Ambystoma tigrinum*, Herrick (1914,

1948) suggested a direct spinotectal projection. In the axolotl, *A. mexicanum*, Nieuwenhuys and Cornelisz (1971) experimentally demonstrated this projection. Our BDA tracing experiments in *P. waltl* show a mainly contralateral spinotectal projection. Some contradictory data were obtained with regard to the origin of this projection. After unilateral HRP injections in *Salamandra salamandra*, Finkenstädt et al. (1983) found spinal projections from the dorsal grey matter of the ipsilateral spinal cord. Rettig (1984, 1988) in *S. salamandra* and *Bolitoglossa subpalmata* as well as Gruberg and Harris (1981) in *A. tigrinum* and *A. mexicanum*, however, found bilateral spinotectal projections from neurons in the ventral horn. With electrophysiological techniques, Gruberg and Harris (1981) observed a rostrocaudal and lateromedial somatosensory body reopresentation in layer 3 of the tectum, below the visual input.

In the present study a sparse spinal innervation of the anteroventral and posterodorsal thalamic zones was observed. A much denser spinothalamic projection was found to the ventral thalamus. In *Ambystoma tigrinum*, Gruberg and Solish (1978) reported the presence of degenerating fibers in the ventral thalamus after spinal hemisections. The ventral thalamus also receives retinal, tectal and tegmental input (Wicht and Himstedt, 1988).

Ventral quadrant pathways in vertebrates

Ventral quadrant pathways comparable to those demonstrated in amphibians are found in other jawed vertebrates. This system of ascending fibers, sometimes called the spinal lemniscus after an ascending system described by Herrick (1948) in *Ambystoma tigrium*, arises in spinal neurons and courses rostrally through the ventralateral spinal cord and brain stem. All vertebrates have in common a distinct spinoreticular pathway passing via the ventral quadrant (Table 3). A ventral spinocerebellar pathways seems to be restricted to gnathostomes. A spinotectal component is variable. Ventral quadrant fibers extend as far as the diencephalon in terrestrial vertebrates and in certain sharks. The ventral quadrant pathways therefore include spinoreticular, spinocerebellar, spinotectal, and spinothalamic tracts.

In agnathans, ventral quadrant pathways extend rostrally, along the ventrolateral border of the spinal and medullary central grey and consists of spinoreticular and possibly spinovestibular projections (Ronan and Northcutt, 1990). In the Pacific hagfish, *Eptatretus stouti*, spinal lemniscal fibers heavily terminate in the mesencephalic tectum, but no spinal projection to the thalamus was found. The spinal lemniscus of lampreys ascends to the isthmus level and may extend into the mesencephalic tegmentum. Retrograde tracer data (Ronan and Northcutt, 1990) indicate that a very small population of cells in the most rostral part of the lamprey spinal cord may project to the tectum and diencephalon. Unlike the spinocerebellar projections of jawed vertebrates which terminate in the cerebellar granule layer, spinal lemniscal fibers in lampreys do not enter the periventricular grey of the cerebellar region (Ronan and Northcutt, 1990).

Ventral quadrant fibers project to the reticular formation, particularly its caudal part, the cerebellar granule layer, and the tectum mesencephalic in the spotted dogfish, *Scyliorhinus canicula* (Hayle, 1973a,b) and the nurse shark, *Ginglymostoma*

cirratum (Ebbesson and Hodde, 1981). In the latter, advanced species, spinal fibers also reach a subtectal, intercollicular zone and the central nucleus of the thalamus. Spinoreticular fibers passing via the ventral quadrant of the spinal cord were demonstrated in representatives of three grades of ray-finned bony fishes: bichirs, *Polypterus palmas* (unpublished observations quoted by Ronan and Northcutt, 1990), gars, *Lepisosteus osseus* (unpublished observations quoted by Ronan and Northcutt, 1990), rudds, *Scardinius erythrophtalmus* (Hayle, 1973a,b), and one genus of lobe-finned bony fish, the African lungfish, *Protopterus* spp. (unpublished observations quoted by Ronan and Northcutt, 1990). The spinoreticular projections diminish rostrally and likely terminate most heavily in the caudal reticular formation. In the ray-finned fishes and probably in African lungfishes, spinal fibers project to the cerebellar granule layer, whereas a projection to the tectum was only observed in lungfishes (unpublished observations quoted by Ronan and Northcutt, 1990). In *Sebastiscus marmoratus*, Murakami and Ito (1985) showed ascending projection of the spinal dorsal horn, via the ventral quadrant of the cord, to the reticular formation, the vagal lobe, octaval nuclei, the cerebellum and the nucleus ventromedialis thalami. Ito et al. (1986) suggested that the spinothalamic projection may arise in the nucleus of the lateral funiculus and, therefore, form a cervicothalamic rather than a proper spinothalamic pathway. In the himé salmon, *Oncorhynchus nerka*, Oka et al. (1986) found, in addition to spinoreticular and spinocerebellar components of the spinal lemniscus, a projection to the lateral part of the torus semicircularis.

Ascending ventral quadrant pathways in reptiles were studied in a number of species - a boid snake, *Constrictor constrictor* (Ebbesson, 1969); turtles, *Pelusios subniger*, *Pelomedusa subrufa*, *Podocnemis unifilis* (Pedersen, 1973), and, especially, *Pseudemys scripta elegans* (Ebbesson, 1969; Künzle and Woodson, 1982); lizards, a lacertid, *Lacerta viridis* (Goldby and Robinson, 1962), the tegu lizard, *Tupinambis teguixin* (Ebbesson, 1967), and the savannah monitor lizard, *Varanus exanthematicus* (Hoogland, 1981; ten Donkelaar et al., 1987), and caimans, *Caiman crocodilus* (Ebbesson and Goodman, 1981; Pritz and Stritzel, 1989). The bulk of the ventral quadrant fibers innervate the rhombencephalic reticular formation, particularly its caudal part. Other rhombencephalic centers such as the nucleus of the solitary tract, the vestibular nuclear complex, the perihypoglossal nuclear complex, the inferior olive, and the area in and around the facial motor nucleus are innervated by spinal fibers. The cerebellum receives an extensive spinal innervation (for retrograde tracer data see: Bangma and ten Donkelaar, 1982; Künzle, 1983). In the mesencephalon, the nucleus intercollicularis, a diffuse cell mass in the dorsolateral part of the caudal mesencephalon, receives an extensive spinal innervation (Ebbesson, 1967, 1969; ten Donkelaar et al., 1987). Evidence for spinal projections to the intercollicular nucleus was found in various vertebrates (RoBards et al., 1976) and is included in Table 3. In mammals, e.g., the North American opossum (Hazlett et al., 1972; RoBards et al., 1976), the intercollicular terminal zone receives afferents from the spinal cord, the dorsal column nuclei, and the somatosensory cortex. In lizards, so far only spinal afferents and a projection from the dorsal column nucleus (Ebbesson, 1978) were demonstrated. Another main mesencephalic target of spinal afferents in reptiles is the laminar nucleus of the torus semicircularis. This cell group

may be comparable to the mammalian periaqueductal grey (ten Donkelaar et al., 1987; Puelles et al., 1994).

Anterograde degeneration studies in lizards (Ebbesson, 1967; Kusuma, 1979; ten Donkelaar et al., 1987) showed the presence of a direct spinothalamic projection, terminating in a poorly differentiated region labeled dorsal intermediate nucleus (Ebbesson, 1967) of the dorsal thalamus. This nucleus may be comparable to the intralaminar nuclei of mammals (Ebbesson, 1967), which receive the so-called paleospinothalamic pathway (Mehler, 1957; Herrick and Bishop, 1958). In *Caiman crocodilus*, Ebbesson and Goodman (1981) demonstrated in addition to the dorsal spinothalamic tract (terminating in the nucleus medialis posterior), a ventral extension of the spinal lemniscus. This ventral pathway terminates in the ventrolateral thalamic nucleus. This connection is reminiscent of the mammalian spinothalamic projection (neospinothalamic tract) to the ventrobasal complex (e.g., Mehler, 1957, 1969). In a retrograde tracer study in *Varanus exanthematicus*, Hoogland (1981) showed that the spinal cord projects to three thalamic areas: 1) an area that includes both the nucleus dorsolateralis and the nucleus intermedius dorsalis; b) the nucleus ventrolateralis; and c) the nucleus dorsomedialis. Moreover, it was demonstrated that these spinothalamic projections arise from different populations of neurons in the spinal cord. Comparable data were obtained in *Pseudemys scripta elegans* (Künzle and Woodson, 1982; Künzle and Schnyder, 1983).

In pigeons (*Columba livia*), ascending spinal projections were studied with anterograde degeneration (Karten, 1963; Karten and Rezvin, 1966;van den Akker, 1970), tract-tracing (Wild, 1983; Funke and Necker, 1986; Necker, 1989, 1990; Schneider and Necker, 1989) and electrophysiological (Delius and Bennetto, 1972; Necker, 1989; Funke, 1989a,b) techniques. Funke and Necker (1986) and Necker (1989, 1990) found that ventral quadrant fibers mainly arise in neurons in the laminae V-VIII of the spinal gray, bilaterally at cervical levels, and contralaterally at lumbar levels. Ascending spinal projections to the raphe and medial reticular targets arise from neurons located in all spinal laminae, except II and IV (Necker, 1989; Schneider and Necker, 1989). In experiments with large HRP applications to the lateral aspect of the reticular formation labeled neurons were found bilaterally, in lamina I and, to a lesser extent, in laminae IV and IX (Necker, 1989). Spinocerebellar fibers form a distinct component of the ventral quadrant fibers (van den Akker, 1970; Vielvoye, 1977; Okado et al., 1987). Sparse data exist in the literature on the spinomesencephalic projections in birds. Spinal projections were demonstrated to the intercollicular nucleus (Necker, 1989) and to the tectum (Karten, 1963). Spinothalamic projections were already observed in degeneration studies (Karten, 1963; Karten and Rezvin, 1966). Later tracing studies revealed lumbar spinothalamic projections to the nucleus dorsointermedius ventralis anterior (DIVA) and to a lesser extent to the nucleus intercalatus thalami, nucleus subrotundus, and the stratum cellulare externum and internum (Schneider and Necker, 1989), whereas cervical spinothalamic projections reach the nucleus dorsolateralis posterior (Schneider and Necker, 1989). The cells of origin of spinothalamic projections were demonstrated in several studies (Wild, 1983; Necker, 1989; Schneider and Necker, 1989). Schneider and Necker (1989) labeled only contralateral lumbar neurons in the intermediate grey in experiments with WGA-HRP applications in DIVA.

Necker (1989) labeled neurons at the base of the dorsal horn, close to the midline, and at the lateral aspect of the neck of the dorsal horn, in dorsolateral thalamic HRP application experiments, and only in those cases with large applications, labeled neurons were observed in the contralateral lumbar laminae V-VI. On the other hand, HRP applications into the nucleus ovoidalis labeled contralateral neurons in the laminae V-VI and VIII.

In mammals, the presence of extensive ventral quadrant pathways is well known (see Mehler, 1969; Willis and Coggeshall, 1991; Tracey, 1995). It includes spinoreticular, spinomesencephalic and spinothalamic projections. Retrograde tracer studies (Kevetter et al., 1982; Kevetter and Willis, 1984; Menétrey et al., 1984; Lima, 1990) showed three main groups of spinoreticular neurons: 1) those projecting to the lateral reticular nucleus; 2) a group projecting to the medial nuclei of the pontomedullary reticular formation, and 3) neurons that innervate the dorsal reticular nucleus. Spinal projections in mammals to the medial reticular formation arise bilaterally, but with a contralateral predominance, in neurons located mainly in laminae V, VII and VIII and, to a lesser extent, in lamina X and at the neck of the dorsal horn, throughout the spinal cord, but more densely at higher spinal segments, where also large ventral spinal neurons give rise to spinoreticular projections. The dorsal reticular nucleus receives projections from lamina I and X as well.

Although some spinomesencephalic projections in mammals ascend in the dorsolateral funiculus, the bulk of the spinal projections to the mesencephalon ascends via the ventrolateral quadrant intermingled with spinoreticular and spinothalamic axons. A rough rostrocaudal somatotopic arrangement was observed in the spinal projections to the mesencephalon. Lumbosacral projections reach more caudal mesencephalic targets than those that arise at cervical levels (Willis and Coggeshall, 1991). Spinomesencephalic projections innervate, among other structures, the nucleus cuneiformis, the parabrachial nucleus, the periaqueductal grey, the intercollicular nucleus and the deep layers of the superior colliculus. The cells of origin of spinomesencephalic projections are located throughout the spinal cord, but in higher concentration at higher spinal levels, in laminae I, III-V, VII, and X, and in the lateral cervical and lateral spinal nuclei (see Willis and Coggeshall, 1991).

The spinothalamic tract in mammals arises from neurons throughout the spinal cord. However, together with the lateral cervical nucleus neurons that also project to the thalamus, the highest concentration of spinothalamic neurons is present in cervical segments (Willis and Coggeshall, 1991). In addition to the spinothalamic projections via the dorsolateral funiculus (dorsolateral spinothalamic tract) arising from laminae I, II and III neurons to the ventral posterolateral and the submedius nuclei and the intralaminar nuclear complex, in the brain of marsupials, carnivores, rodents and primates two distinct components of spinothalamic projections via the ventral quadrant exist, 1) a mainly contralateral projection from laminae IV-VI neurons to the ventral posterolateral and the central lateral nuclei, and 2) a projection from laminae VII-X neurons to the contralateral posterior nuclear complex and the central lateral nucleus (Willis and Coggeshall, 1991).

Kevetter and Willis (1984) reviewed the then available data on the collateralization of the ascending projections in the ventral quadrant of the mammalian spinal cord as well as the formation of direct spinothalamic projections throughout phylogeny. They showed spinal collateral projections to the medial (intralaminar) and lateral (ventrobasal) thalamus considered as paleospinothalamic and neospinothalamic tracts, respectively (Mehler, 1969). Additionally, ascending collateral projections to the spinal cord and the medullary reticular formation, thalamus and reticular formation, intralaminar thalamus, ventrobasal thalamus and reticular formation were reported in different mammalian species (Kevetter and Willis, 1984). Moreover, later studies demonstrated that spinal neurons projecting to the mesencephalic periaqueductal gray send axon collaterals to the medullary reticular formation (Pechura and Liu, 1986) or the ventrobasal complex of the thalamus (Harmann et al., 1988). In our amphibian material we could observe thin fibers that innervate the medullary reticular formation as collateral branches of thicker ascending fibers located peripherally in the rhombencephalon that ascend to more rostral regions. However, retrograde double labeling studies are needed to confirm whether these fibers reach more rostral mesencephalic or diencephalic targets. As far as we know, so far no double-labeling studies demonstrated the collateralization of ascending spinal projections in non-mammalian vertebrates.

Based on previous anterograde degeneration (Hayle, 1973a; Ebbesson, 1969; Ebbesson and Hodde 1981) and tracing (Neary and Wilczynski, 1977, 1979) studies, direct spinothalamic projections, althought present in cartilaginous fishes (Ebbesson and Hodde, 1981), were viewed by Kevetter and Willis (1984) as an advanced evolutionary character only present in the brains of amniotes. The spinothalamic projection in some elasmobranchs was viewed as a secondary obtained character. However, later studies in agnathans (Ronan and Northcutt, 1990), teleosts (Murakami and Ito, 1985; Ito et al., 1986) and amphibians (A. Muñoz et al., 1994a; the present study) confirmed the existence of direct spinothalamic projections suggesting a common pattern throughout all the vertebrate classes.

In reptiles, the existence of a paleospinothalamic pathway to the dorsolateral and the intermediodorsal thalamic nuclei, the probable homologue of the intralaminar and posterior thalamus of mammals, and a neospinothalamic pathway to the ventrolateral thalamus, equivalent to the mammalian ventrobasal complex were shown (Ebbesson, 1967, 1978; Riss et al., 1972; Pedersen, 1973; Northcutt and Pritz, 1978; Ebbesson and Goodman, 1981; Hoogland, 1981) that arise from different spinal neuronal populations (Hoogland 1981). Our amphibian material (A. Muñoz et al., 1994a; the present study) revealed spinal projections to the ventral thalamus and, more sparsely, to different dorsal thalamic nuclei allowing comparison to the spinothalamic projections demonstrated in the brains of reptiles and mammals. In reptiles, spinal fibers that innervate dorsal and ventral thalamic nuclei enter the diencephalon dorsomedially or ventrolaterally, respectively (Ebbesson, 1967; Pedersen, 1973; Hoogland, 1981). Spinothalamic fibers to the ventral thalamus in amphibians enter the diencephalon ventrolaterally (A. Muñoz et al., 1994a; the present study). However, due to the low density of spinal fibers present in the dorsal thalamus, the way of entrance of these fibers could not be determined. However, since some fibers were observed at the

pretectal gray at the mesodiencephalic junction it seems likely that they enter the diencephalon dorsally. Additionally, different spinal neuronal populations giving rise to dorsal and ventral thalamic projections could not be distinguished since tracer applications restricted to dorsal thalamic nuclei failed to label neurons at the spinal cord (Neary and Wilczynski, 1977; 1979, own unpublished observations), likely due to the low density of dorsal spinothalamic projection. Studies in amphibians and reptiles, therefore strongly suggest the presence of ventral thalamic nuclei reminiscent of the mammalian ventrobasal complex. Their ascending projections are only incompletely known, and form the subject of future studies.

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Table 1 Dorsal funicular pathways in vertebrates (based in part on Ronan and Northcutt, 1990)

Species	Target at obex level (DCN)	Octavolateral target	Cerebellar projection	PDCS	Refs.
Agnathans					
Lampreys					
- Sea lamprey (*Petromyzon marinus*)	+	?	+	?	19, 23
- Silver lamprey (*Ichthyomyzon unicuspis*)	+	?	+	?	2, 23
Hagfishes					
- Pacific hagfish (*Eptatretus stouti*)	+	?	?	?	23
Gnathostomes					
Cartilaginous fishes					
- Spotted dogfish (*Scyliorhinus canicula*)	?	?	?	?	9
- Nurse shark (*Ginglymostoma cirratum*)	+	+	+	?	7
Bony fishes					
- Bichir (*Polypterus palmas*)	+	+	-	?	23
- Gar (*Lepisosteus osseus*)	+	+	-	?	23
- Himé salmon (*Oncorhynchus nerka*)	+	-	-	?	20
- Lungfish (*Protopterus* species)	+	+	+	?	23
Amphibians					
Urodeles					
- Lungless salamanders	+	+	?	?	24
- Axoloti (*Ambystoma mexicanum*)	+	+	-	-	18
- Ribbed newt (*Pleurodeles waltl*)	+	+	+	+	17
Anurans					
- *Rana esculenta*	+	+	+	?	1, 25
- *R. temporaria*	+	+	+	?	9, 10
- *R. catesbeiana*	+	+	+	?	4, 5
- *R. perezi*	+	+	+	+	16, 17
- *Xenopus laevis*	+	+	+	+	16, 17, 26
Reptiles					
Turtles					
- *Pelusios subniger*	+	+	+	?	21
- *Pelomedusa subrufa*	+	+	+	?	21
- *Podocnemis unifilis*	+	+	+	?	21
- *Pseudemys scripta elegans*	+	+	+	?	4, 14
Lizards					
- *Lacerta viridis*	+	+	+	?	11
- *Tupinambis teguixin*	+	+	+	?	3
Snakes					
- *Constrictor constrictor*	+	+	+	?	4
- *Thamnophis sirtalis*	+	+	-	?	12
Crocodilians					
- *Caiman crocodilus*	+	+	+	+	6, 22
Birds					
- Pigeon (*Columba livia*)	+	-	-	+	8, 13, 27, 28
Mammals	+	+	-	+	29

Symbols used: + present; - not reported; ? unknown or questionable.
References: 1 - Antal et al., 1980; 2 - Dubuc et al., 1993; 3 - Ebbesson, 1967; 4 - Ebbesson, 1969; 5 - Ebbesson, 1976; 6 - Ebbesson and Goodman 1981; 7 - Ebbesson and Hodde, 1981; 8 - Funke, 1988; 9, 10 - Hayle, 1973a,b; 11 - Jacobs, 1968; 12 - Jacobs and Sis, 1980; 13 - Karten, 1963; 14 - Künzle, 1982; 15 - Künzle and Woodson, 1983; 16 - A. Muñoz et al., 1995b; 17 - A. Muñoz et al., present study; 18 - Nieuwenhuys and Cornelisz, 1971; 19 - Northcutt and Ebbesson, 1980; 20 - Oka et a., 1986; 21 - Pedersen, 1973; 22 - Pritz and Stritzel, 1994; 23 - Ronan and Northcutt, 1990; 24 - Roth and Wake, 1985; 25 - Székely et al., 1980; 26 - ten Donkelaar and de Boer-van Huizen, 1991; 27 - van den Akker 1970; 28 - Wild, 1985; 29 - Willis and Coggeshall, 1991.

Table 2 Dorsolateral funicular pathways in vertebrates

Species	Spinocervical tract	Target at spinomedullary junction (LCN)	Projections to			Refs.
			nucleus of the solitary tract	lateral reticular zone	para-brachial area	
Agnathans						
Lampreys						
- Sea lamprey *(Petromyzon marinus)*	•	•	?	?	?	18, 19
- Silver lamprey *(Ichthyomyzon unicuspis)*	•	?	?	?	•	19
Hagfishes						
- Pacific hagfish *(Eptatretus stouti)*	•	?	?	?	?	19
Gnathostomes						
Cartilaginous fishes						
- Spotted dogfish *(Scyliorhinus canicula)*	•	-	-	-	-	7
- Nurse shark *(Ginglymostoma cirratum)*	•	?	?	?	?	3
Bony fishes						
- Sea robin *(Prionotus carolinus)*	+	?	?	?	?	4
- *Sebastiscus marmoratus*	?	+	?	?	?	10
- Rudd *(Scardinius erythrophthalmus)*	•	-	-	-	-	7, 8
Amphibians						
- Tiger salamander *(Ambystoma tigrinum)*	+	?	?	?	?	9
- Ribbed newt *(Pleurodeles waltl)*	+	+	+	+	+	15
- Clawed toad *(Xenopus laevis)*	+	+	+	+	+	12-15
- Large green frog *(Rana perezi)*	+	+	+	+	+	12, 13, 15
Reptiles						
- Red-eared turtle *(Pseudemys scripta elegans)*	•	?	+	•	?	2, 11
- Tegu lizard *(Tupinambis teguixin)*	+	+	+	?	•	1
Birds						
- Pigeon *(Columba livia)*	+	+	+	?	?	5, 6, 16, 17, 20
Mammals	+	+	+	+	+	21

Symbols used: + positive evidence; • suggestive evidence; - not reported; ? unknown or questionable.
References: 1 - Ebbesson, 1967; 2 - Ebbesson, 1969; 3 - Ebbesson and Hodde, 1981; 4 - Finger, 1981; 5 - Funke, 1988; 6 - Funke and Necker 1986; 7, 8 - Hayle, 1973a,b; 9 - Herrick, 1930; 10 - Ito et al., 1986; 11 - Künzle and Woodson, 1982; 12-14, A. Muñoz et al., 1995a,b, 1996; 15 - A. Muñoz et al., present study; 16, 17 - Necker 1989, 1991; 18 - Northcutt and Ebbesson, 1980; 19 - Ronan and Northcutt, 1990; 20 - van den Akker , 1970; 21 - Willis and Coggeshall, 1991.

Table 3 Ventral quadrant pathways in vertebrates (based in part on Ronan and Northcutt, 1990)

Species	Spinoreticular component	Spinocerebellar component	Projection to torus or PAG	Spinotectal component	Spinothalamic component	Refs.
Agnathans						
Lampreys						
- Sea and silver lampreys	+	?	-	•	•	20, 22
Hagfishes						
- Pacific hagfish *(Eptatretus stouti)*	+	-	-	+	-	22
Gnathostomes						
Cartilaginous fishes						
- Spotted dogfish *(Scyliorhinus canicula)*	+	+	?	+	-	7, 8
- Nurse shark *(Ginglymostoma cirratum)*	+	+	?	+	+	5
Bony fishes						
- Bichir *(Polypterus palmas)*	+	+	?	-	-	22
- Gar *(Lepisosteus osseus)*	+	+	?	-	-	22
- Himé salmon *(Oncorhynchus nerka)*	+	+	+	-	-	21
- *Sebastiscus marmoratus*	+	+	+	-	+	17
- Lungfish *(Protopterus species)*	+	+	?	+	-	22
Amphibians						
Urodeles						
- Axolotl *(Ambystoma mexicanum)*	+	+	?	?	-	19
- Tiger salamander *(A. tigrinum)*	+	+	+	+	+	9
- Ribbed newt *(Pleurodeles waltl)*	+	+	+	+	+	15, 16
Anurans						
- *Rana temporaria*	+	+	?	-	-	7, 8
- *R. catesbeiana*	+	+	+	-	?	3
- *R. perezi*	+	+	+	+	+	15, 16
- *Xenopus laevis*	+	+	+	+	+	16
Reptiles						
Turtles						
- *Pseudemys scripta elegans*	+	+	+	+	+	2, 12, 13
Lizards						
- *Tupinambis teguixin*	+	+	+	+	+	1
- *Varanus exanthematicus*	+	+	+	+	+	10, 24
Snakes						
- *Constrictor constrictor*	+	+	+	+	+	2
Crocodilians						
- *Caiman crocodilus*	+	+	+	+	+	4
Birds						
- Pigeon *(Columba livia)*	+	+	+	+	+	6, 11, 18, 23
Mammals	+	+	+	+	+	14, 25

Symbols used: + positive evidence; • suggestive evidence; - not reported; ? unknown or questionable.
References: 1, 2, 3 - Ebbesson, 1967, 1969, 1976; 4 - Ebbesson and Goodman, 1981; 5 - Ebbesson and Hodde, 1981; 6 - Funke and Necker 1986; 7, 8 - Hayle, 1973a,b; 9 - Herrick, 1930; 10 - Hoogland, 1981; 11 - Karten, 1963; 12 - Künzle and Schnyder, 1983; 13 - Künzle and Woodson, 1982; 14 - Mehler, 1969; 15 - A. Muñoz et al., 1994a; 16 - A. Muñoz et al., present study; 17 - Murakami and Ito, 1985; 18 - Necker, 1989; 19 - Nieuwenhuys and Cornelisz, 1971; 20 - Northcutt and Ebbesson, 1980; 21 - Oka et al 1986; 22 - Ronan and Northcutt, 1990; 23 - Schneider and Necker, 1989; 24 - ten Donkelaar et al., 1987; 25 - Willis and Coggeshall, 1995.

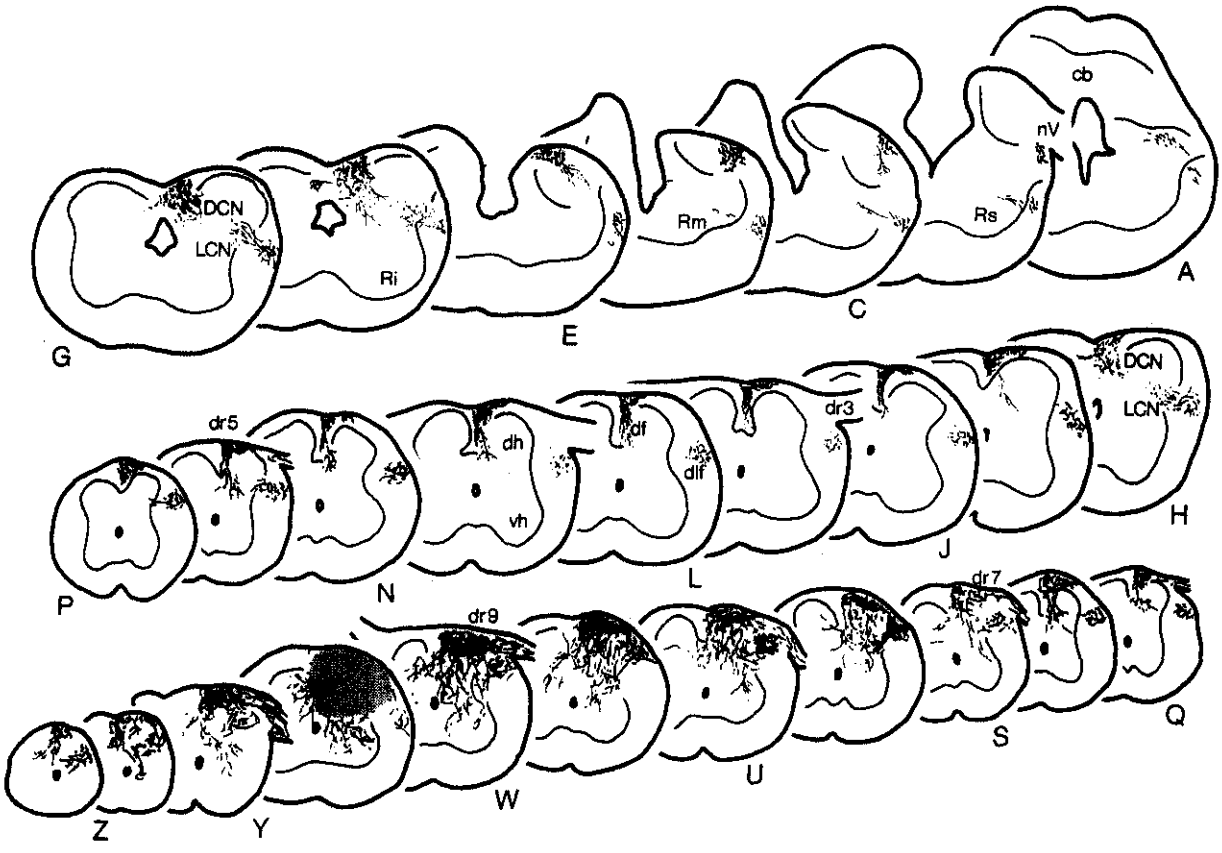

Figure1: Schematic drawing of a series of transverse sections through the brain stem and spinal cord of a young adult *Xenopus laevis* showing the labeling in the dorsal and dorsolateral funiculi after *in vitro* application of 3kD BDA into the lumbar spinal cord between the 9th and 10th dorsal roots.

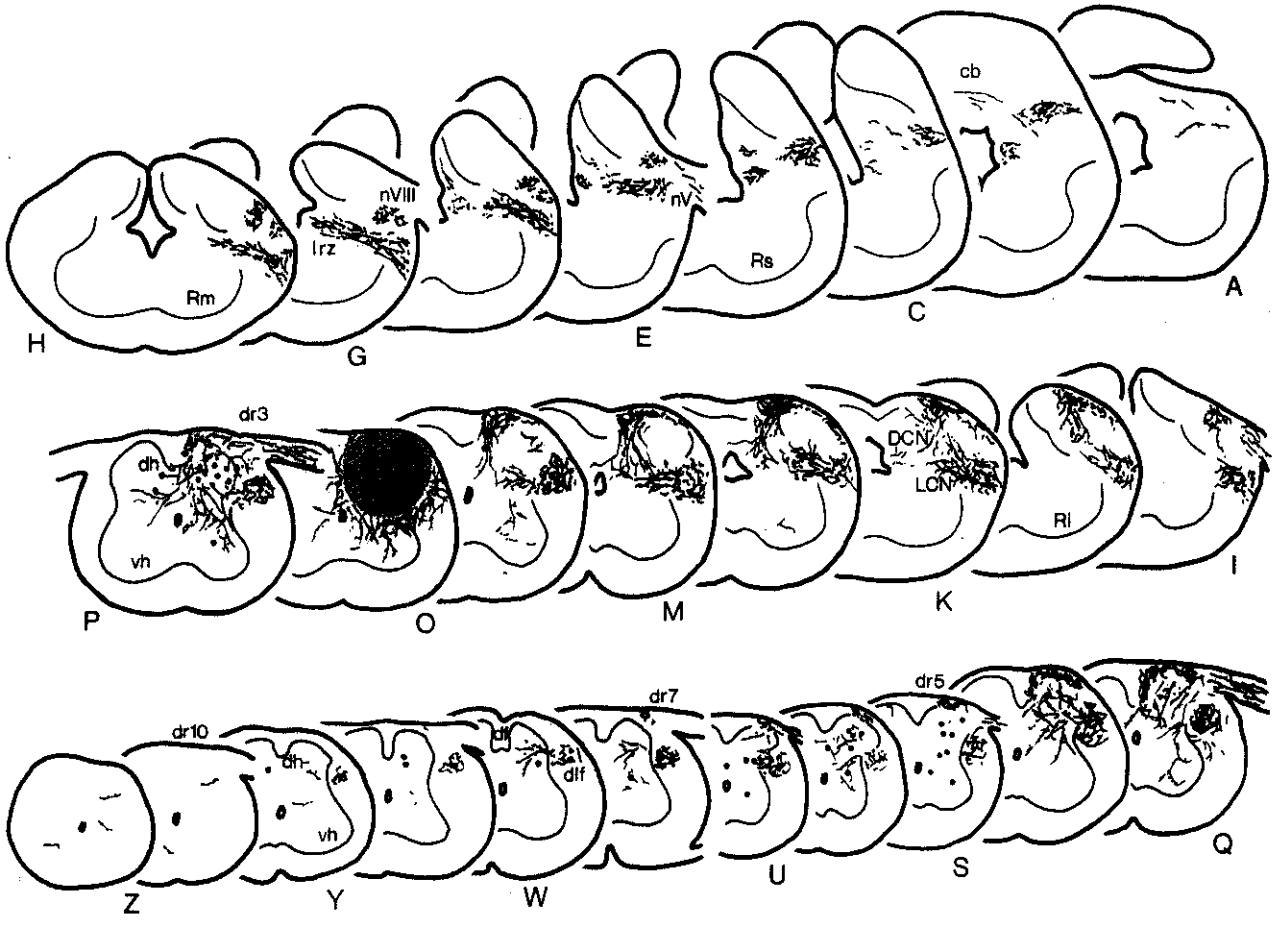

Figure 2: Schematic drawing of a series of transverse sections through the brain stem and spinal cord of a young adult *Xenopus laevis* showing the labeling in the dorsal and dorsolateral funiculi after *in vitro* application of 3kD BDA into the cervical spinal cord, just rostral to the third dorsal root.

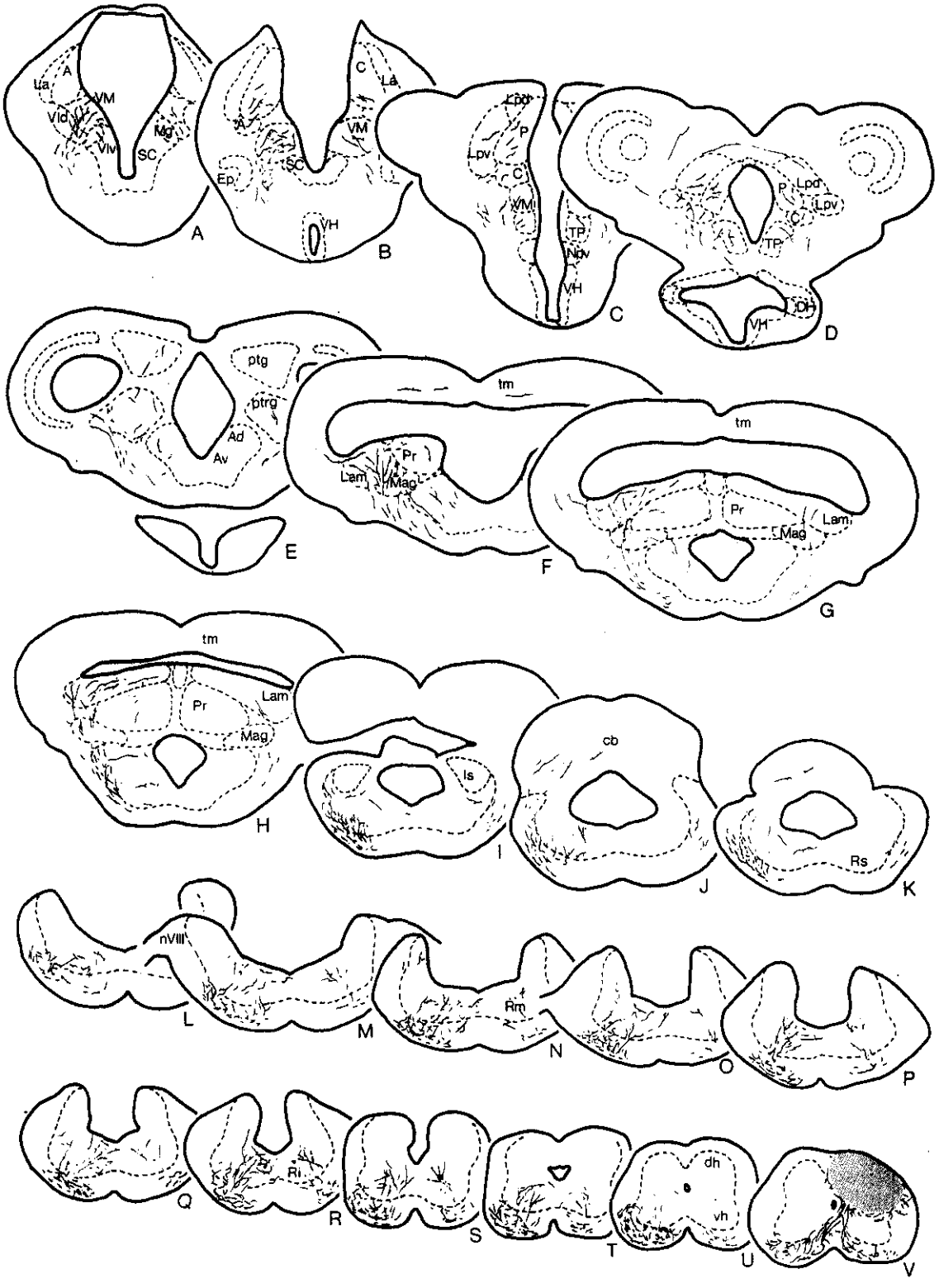

Figure 3: Schematic drawing of a series of transverse sections through the diencephalon (A-D), the mesencephalon (E-H), the rhombencephalon (I-S), and spinal cord (T-V) of *Rana perezi* showing the labeling of the ventral quadrant system after *in vivo* 10kD BDA application to the cervical spinal cord.

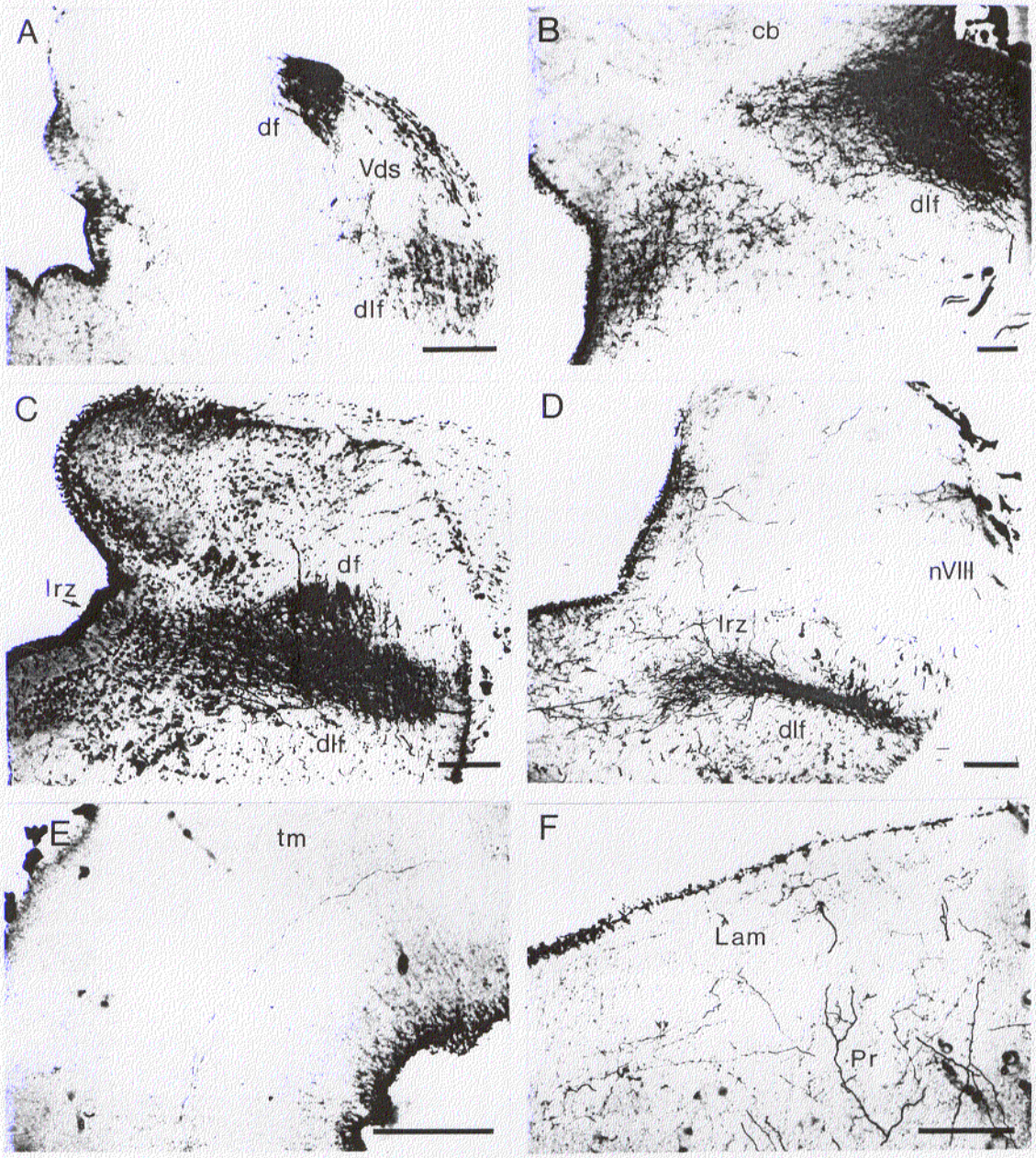

Figure 4: Photomicrographs illustrating the labeling observed in various BDA experiments in anurans. A, Ipsilateral labeling in the dorsal and dorsolateral funiculi as well as of some fibers of the descending tract of the trigeminal nerve, *Xenopus laevis*, thoracic *in vitro* 3kD BDA application; B, ipsilateral labeling of dorsolateral funicular fibers in the subcerebellar regions, *X. laevis*, cervical *in vitro* 3kD BDA application; C, ipsilateral labeling of dorsal and dorsolateral funicular fibers at the level of the eighth cranial nerve root arborizing in the lateral reticular zone, *X. laevis*, cervical *in vitro* 3kD BDA application; D, ipsilateral labeling of dorsolateral funicular fibers at the level of the eighth cranial nerve root arborizing in the lateral reticular zone, *Rana perezi*, cervical *in vivo* 10kD BDA application; E, F, contralateral labeling of ventral quadrant fibers in the tectum mesencephali and torus semicircularis, respectively; *R. perezi*, cervical *in vivo* 10kD BDA application. Scale bars indicate 100 μm.

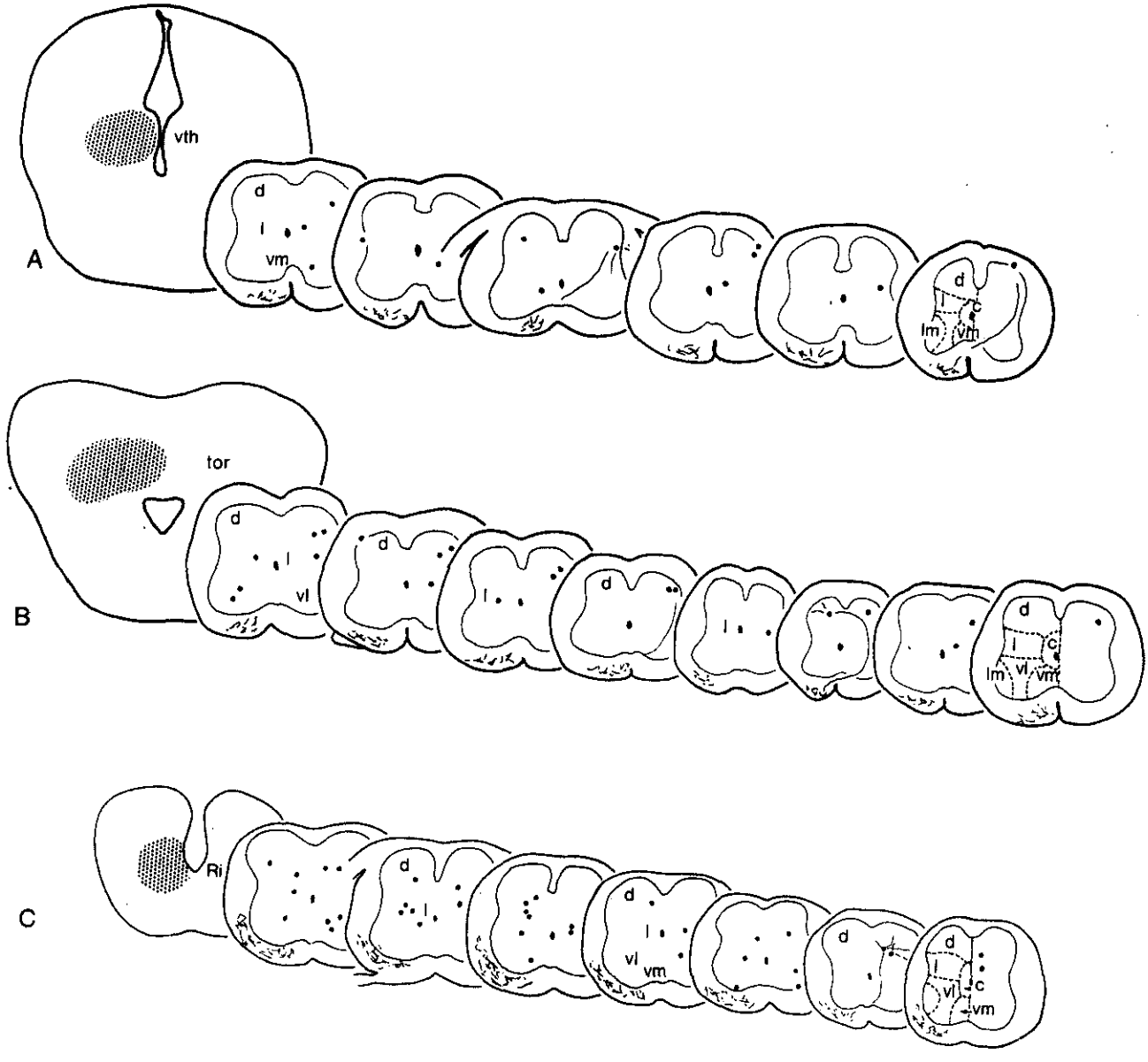

Figure 5: Schematic drawings illustrating the distribution of retrogradely labeled neurons in the spinal cord of *Xenopus laevis* following *in vitro* 3 kD BDA applications to the ventral thalamus (A), the torus semicircularis (B), and the inferior reticular nucleus (C). Examples of labeled neurons are shown in Fig. 6.

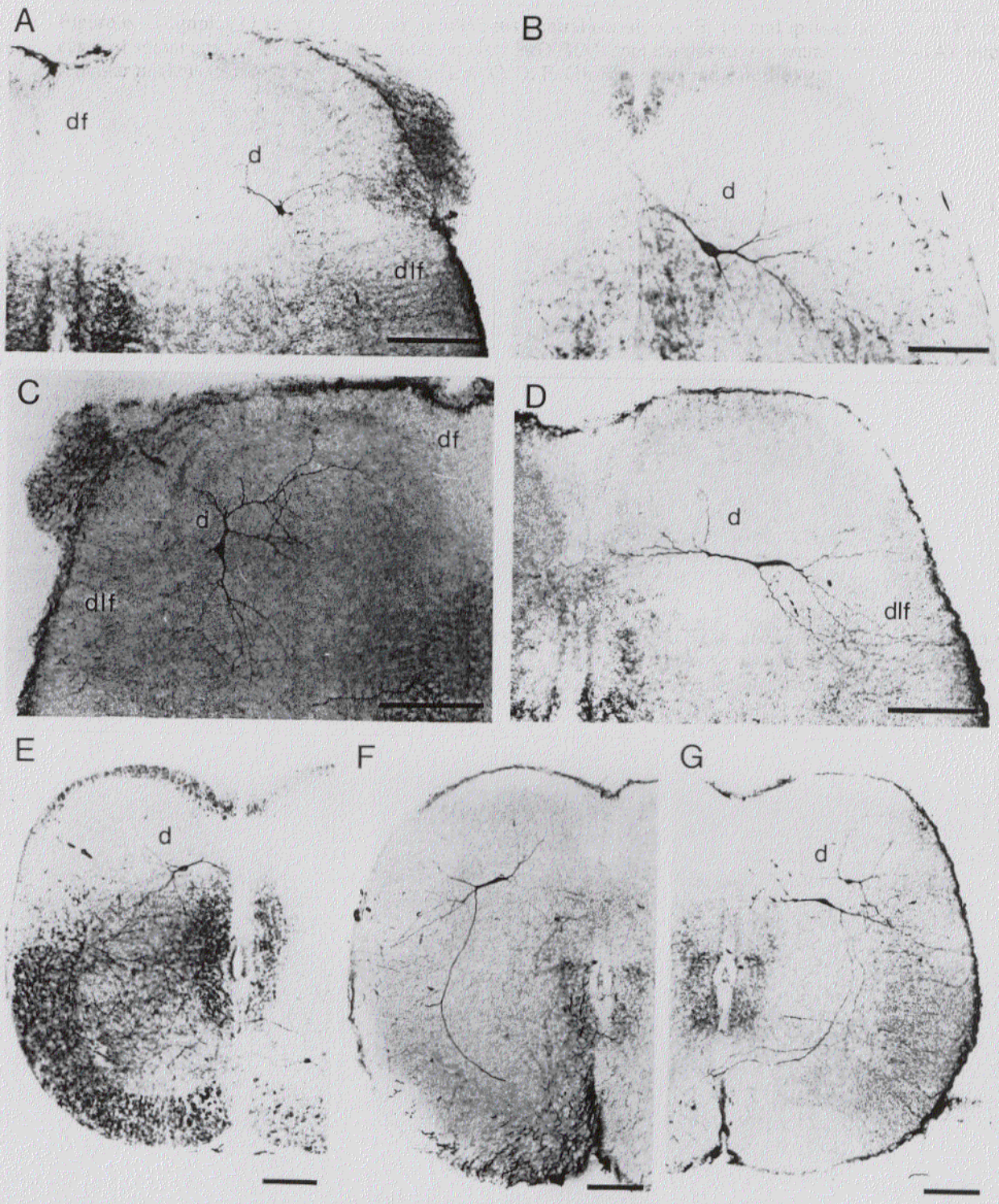

Figure 6: Examples of retrogradely labeled neurons contralaterally (A, B, D) and ipsilaterally (C, E, F) in the cervical spinal cord of *Xenopus laevis* after *in vitro* 3kD BDA applications to the ventral thalamus (A), inferior reticular nucleus (B, E) and torus semicircularis (C, D, F, G). Scale bars indicate 100 µm.

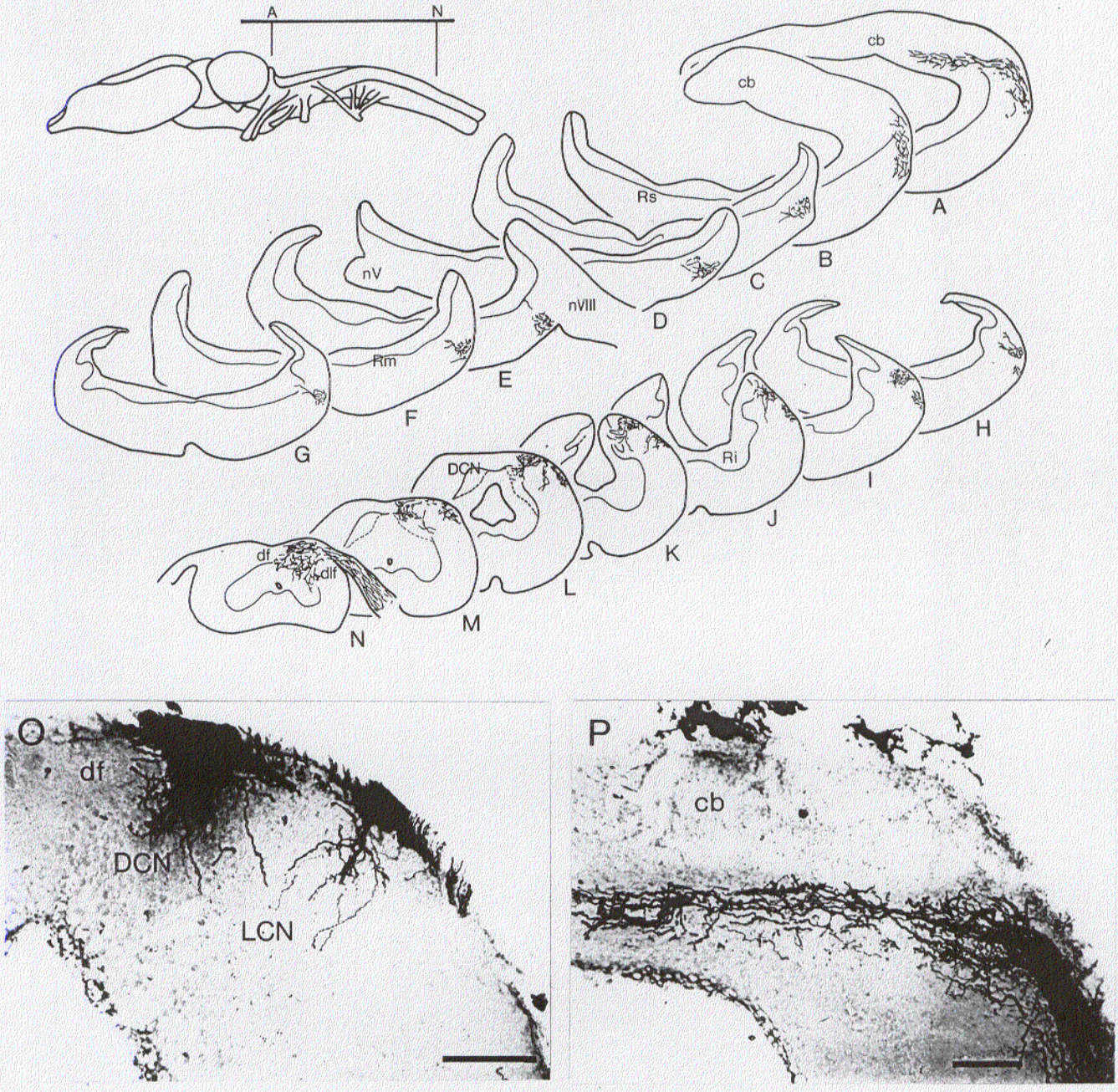

Figure 7: Schematic drawing of a series of transverse sections through the brain stem and spinal cord of *Pleurodeles waltl* showing the distribution of 10kD BDA labeled brachial dorsal root fibers. The levels of the sections are indicated along a dorsal view of the central nervous system of *P. waltl*. Insets show labeling in the ipsilateral dorsal column and lateral cervical nuclei (O), and in the cerebellum (P). Scale bars indicate 100 µm.

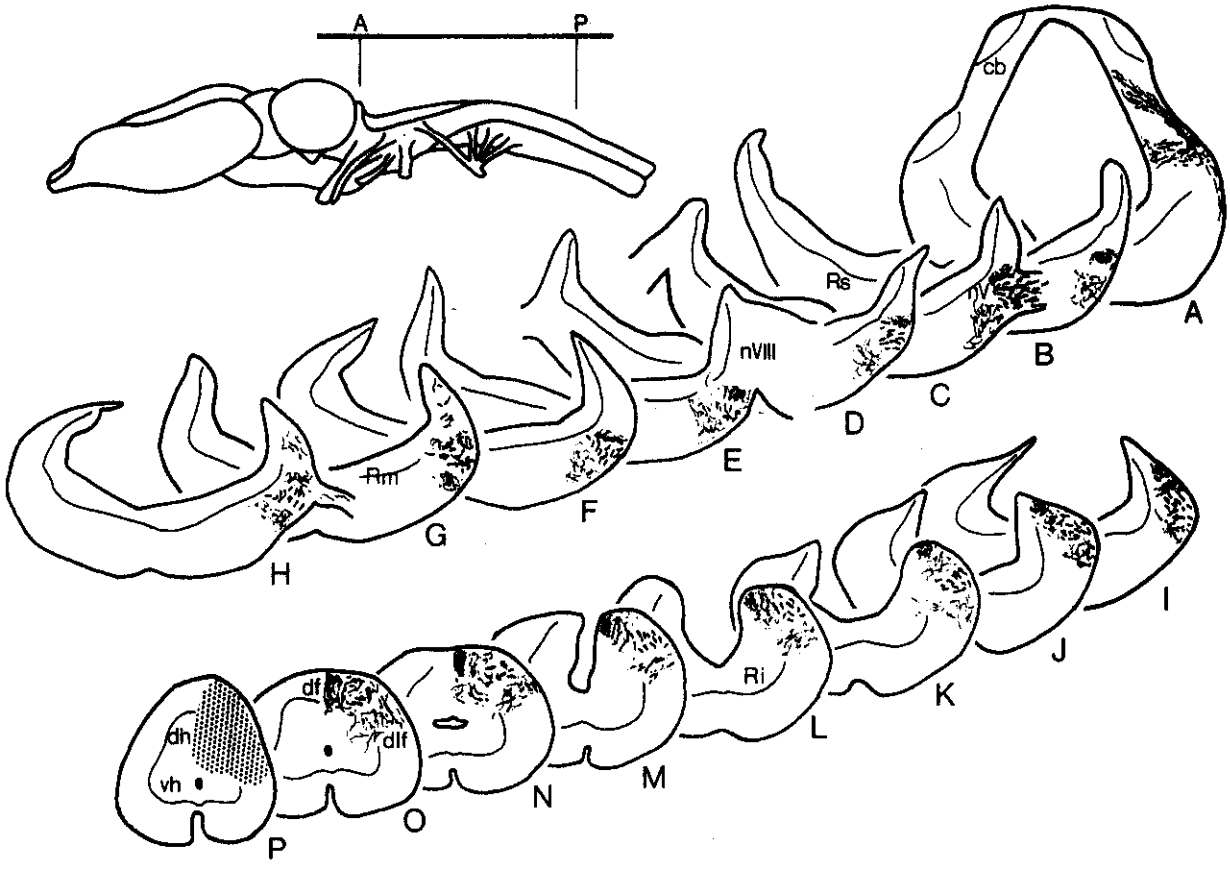

Figure 8: Schematic drawing of a series of transverse sections through the brain stem and spinal cord of *Pleurodeles waltl* showing the labeling of dorsal and dorsolateral funicular components after an *in vivo* 10kD BDA application to the cervical spinal cod. The levels of the sections are indicated along a dorsal view of the central nervous system of *P. waltl*.

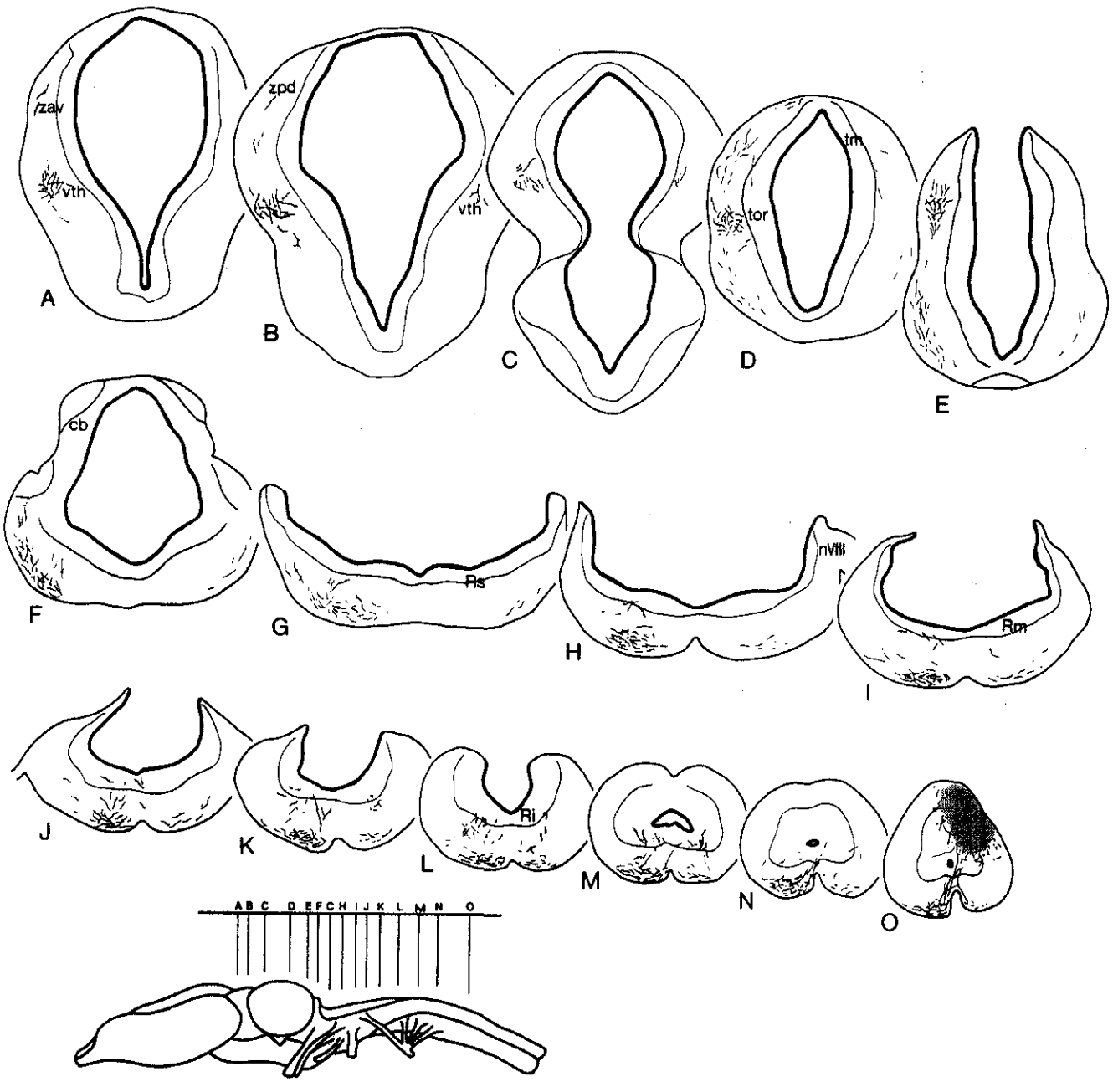

Figure 9: Schematic drawing of a series of transverse sections through the diencephalon (A-C), mesencephalon (D, E), rhombencephalon (F-L), and spinal cord (M-O) of *Pleurodeles waltl* showing the labeling of the ventral quadrant system after *in vivo* 10kD BDA application to the cervical spinal cord.

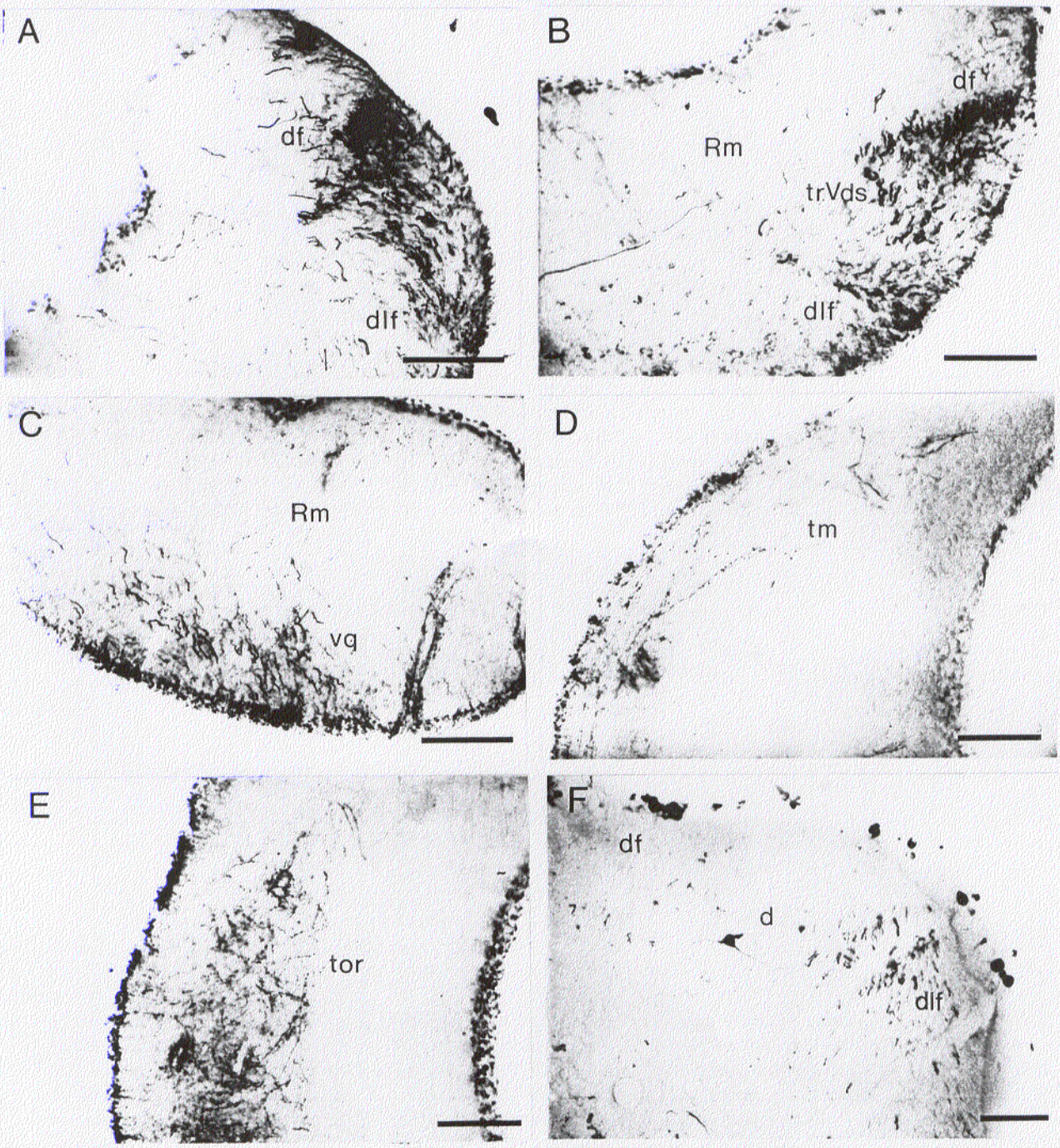

Figure 10: Photomicrographs illustrating the labeling observed in various BDA experiments in *Pleurodeles waltl*. A, Ipsilateral labeling in the dorsal and dorsolateral funiculi in the caudal rhombencephalon after a cervical BDA application; B, ipsilateral labeling at the level of the nucleus reticularis medius, cervical BDA application; C, D and E show contralaterally labeled ventral quadrant fibers innervating the reticular formation (C), the tectum mesencephali (D) and the torus semicircularis (E). In F, a neuron retrogradely labeled from the ventral thalamus in the dorsal horn of the cervical spinal cord is shown. Scale bars indicate 100 µm.

LIST OF ABBREVIATIONS

A	anterior thalamic nucleus
Ad	anterodorsal tegmental nucleus
Av	anteroventral tegmental nucleus
C	central thalamic nucleus
c	central spinal field
cb	cerebellum
d	dorsal spinal field
dr (3-10)	dorsal root 3-10
DCN	dorsal column nucleus
df	dorsal funiculus
DH	dorsal hypothalamic nucleus
dh	dorsal horn
dlf	dorsolateral funiculus
Ep	posterior entopeduncular nucleus
Is	nucleus isthmi
l	lateral spinal field
La	lateral thalamic nucleus, anterior division
Lam	laminar nucleus of the torus semicircularis
LCN	lateral cervical nucleus
lm	lateral motor field
Lpd	lateral thalamic nucleus, posterodorsal division
Lpv	lateral thalamic nucleus, posteroventral division
lrz	lateral reticular zone
Mag	magnocellular nucleus of the torus semicircularis
Mg	magnocellular preoptic nucleus
nV	nervus trigeminus
nVIII	nervus octavus
NPv	nucleus of the periventricular organ
P	posterior thalamic nucleus
Pr	principal nucleus of the torus semicircularis
ptg	pretectal grey
ptrg	pretoral grey
Ri	nucleus reticularis inferior
Rm	nucleus reticularis medius
Rs	nucleus reticularis superior
SC	suprachiasmatic nucleus
tm	tectum mesencephali
tor	torus semicircularis
TP	posterior tuberculum
trVds	descending tract of the trigeminal nerve
VH	ventral hypothalamic nucleus
vh	ventral horn
VLd	ventrolateral thalamic nucleus, dorsal part
VLv	ventrolateral thalamic nucleus, ventral part
vl	ventrolateral spinal field
VM	ventromedial thalamic nucleus
vm	ventromedial spinal field
vth	ventral thalamus
zav	zona anteroventralis of the dorsal thalamus
zpd	zona posterodorsalis of the dorsal thalamus
Vds	nucleus of the descending tract of the trigeminal nerve

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Spinothalamic projections in amphibians as revealed with anterograde tracing techniques

Spinal ascending pathways in amphibians: cells of origin and main targets

COMENTARIOS

3.3

En vertebrados terrestres se ha considerado la existencia de dos sistemas de proyecciones espinales ascendentes: 1) El sistema columna dorsal-lemnisco medial formado por proyecciones espinales primarias y no primarias que, a través del **funículo dorsal,** alcanzan los núcleos de la columna dorsal, los cuales originan el lemnisco medial que asciende hasta el tálamo. 2) El sistema ventrolateral formado por proyecciones secundarias que ascienden inicialmente a través del **cuadrante ventral,** para alcanzar la formación reticular, el mesencéfalo y el tálamo (Willis y Coggeshall, 1991). Además, en mamíferos (Willis y Coggeshall, 1991) se considera un tercer sistema, denominado espino-cervico-talámico, constituído por las proyecciones que ascienden en el **funículo dorsolateral** como el tracto espinocervical que termina en el núcleo cervical lateral, el cual a su vez proyecta contralateralmente al mesencéfalo y al tálamo a través del lemnisco medial. Otras proyecciones que ascienden a través del funículo dorsolateral, alcanzan diversas dianas en el tronco cerebral.

Los datos sobre las proyecciones espinales ascendentes en anfibios, disponibles en la bibliografía, proceden de estudios basados en tinciones argénticas en urodelos (Herrick, 1914, 1930; Herrick y Bishop, 1958) y en técnicas degenerativas tanto en anuros (Ebbesson, 1969, 1976; Hayle, 1973) como en urodelos (Nieuwenhuys y Cornelisz, 1971; Wicht y Himstedt, 1988). En los citados trabajos se ha propuesto la existencia de proyecciones espinales a través de los funículos dorsal, dorsolateral y ventrolateral. Sin embargo, en ocasiones únicamente se han presentado como dos sistemas ascendentes. Además, existen en ellos diferencias en cuanto a la nomenclatura, así como a las proyecciones propuestas en cada uno de dichos sistemas. Recientemente se ha descrito en anuros el sistema espino-cervico-talámico (capítulo 5 de la presente memoria), lo que sugiere que, en vertebrados anamniotas, las proyecciones espinales ascendentes están igualmente organizadas en tres sistemas.

La presencia de vías espinotalámicas en el funículo ventrolateral, parece ser un carácter común y compartido en el cerebro de las distintas clases de amniotas. Así, se han demostrado ampliamente aferencias talámicas desde neuronas espinales en mamíferos (Willis y Coggeshall, 1991), aves (Scheneider y Necker, 1989), y reptiles (Ebbesson, 1967; 69; Künzle y Woodson, 1982). En anamniotas únicamente se describieron proyecciones espinotalámicas en una especie de tiburón galeomorfo (*Ginglymostoma cirratum*) (Ebbesson y Hodde, 1981), y fueron consideradas como un carácter no homólogo a las vías espinotalámicas de amniotas, que evolucionó independientemente (Kevetter y Willis, 1984; Ronan y Northcutt, 1990). En anfibios, hasta el momento, no se habían descrito proyecciones espinotalámicas directas, quizás debido a las limitaciones propias de las técnicas degenerativas (Ebbesson, 1969, 1976; Nieuwenhuys y Cornelisz, 1971; Hayle, 1973; Wicht y Himstedt, 1988).

En el presente capítulo se incluyen dos artículos en los que se ha estudiado la organización de las proyecciones ascendentes espinales en los anuros *Rana perezi* y *Xenopus laevis*, y en el urodelo *Pleurodeles waltl*, mediante la utilización de técnicas de trazado anterógrado y retrógrado, tanto *in vivo* como *in vitro*, utilizando los trazadores peroxidasa de rábano (HRP), leucoaglutinina de *Phaseolus vulgaris* (PHA-L) o dextrano amina combinada con biotina (BDA). En el primer artículo se presentan evidencias

de la existencia de proyecciones espinotalámicas en anfibios, mediante trazado anterógrado. En el segundo artículo se demuestra la organización de las proyecciones espinales ascendentes en tres sistemas fundamentales, funículo dorsal, funículo dorsolateral, y cuadrante ventral (funículos ventral y ventrolateral); caracterizándose los principales centros a los que proyectan, así como las células que originan algunos de ellos, y estableciéndose una comparación, desde un punto de vista evolutivo, con los datos disponibles de otros grupos de vertebrados.

Proyecciones incluídas en el funículo dorsal (DF)

En los experimentos con aplicaciones de diversos trazadores en el asta dorsal, en distintos niveles espinales en *Rana perezi* y en *Xenopus laevis*, observamos un patrón de marcaje de proyecciones ascendentes que terminan en el núcleo de la columna dorsal (ver capítulo 5 de la presente memoria), similar al descrito para las ramas ascendentes de las aferencias primarias espinales (Joseph y Whitlock, 1968a, Antal y cols., 1980; Nikundiwe y cols., 1982, M. Muñoz y cols., 1991; A. Muñoz y cols., 1995b), y a las proyecciones ascendentes no primarias que forman el sistema postsináptico de la columna dorsal (PDCS) (ten Donkelaar y de Boer van Huizen, 1991; A. Muñoz y cols., 1995b). Algunas fibras inervan en dicho nivel el polo caudal del núcleo del tracto solitario y la formación reticular. Coincidiendo con la descripción de las aferencias primarias espinales (Joseph y Whitlock, 1968a, Antal y cols., 1980; Nikundiwe y cols., 1982, M. Muñoz y cols., 1991; A. Muñoz y cols., 1995b), un componente de fibras continúa rostralmente, dando fibras terminales en la formación reticular rombencefálica, núcleo del tracto descendente del nervio trigémino, área octavolateral y capa granular del cerebelo. Sin embargo, hay que señalar que algunas de estas fibras podría corresponder igualmente, a proyecciones no primarias originadas en la médula espinal.

En urodelos existen muy pocos datos en la bibliografía en cuanto a la organización de las aferencias primarias espinales. En nuestros experimentos, con aplicaciones de BDA en raíces dorsales cervicales y lumbares en *Pleurodeles waltl*, hemos observado un patrón de organización similar a nuestros estudios en anuros. Las aferencias primarias espinales, al entrar en la médula espinal, establecen un componente medial cuyas fibras ascienden y descienden en el DF y un componente lateral, equivalente al tracto de Lissauer, que discurre en el funículo dorsolateral. Las fibras del componente medial ascienden en el DF somatotópicamente organizadas, de manera que las aferencias lumbares y cervicales discurren medial y lateralmente en el DF respectivamentre, y alcanzan de la misma forma el núcleo de la columna dorsal (ver capítulo 6 de la presente memoria). Las aferencias lumbares más rostrales se observan en niveles ligeramente anteriores al núcleo de la columna dorsal, mientras que las cervicales continuan ascendiendo a lo largo del rombencéfalo, dorsalmente al tracto descendente del nervio trigémino, para terminar en la capa granular del cerebelo. Dichos resultados coinciden con la distribución de las aferencias braquiales descrita por Roth y Wake (1992) en salamandras de la familia *Plethodontidae*, si bien dichos autores no observaron proyecciones directas al cerebelo.

En nuestros experimentos en *Pleurodeles waltl* con aplicaciones de BDA en el asta dorsal

espinal, en niveles cervicales, se observaron en el DF dos componentes de fibras ascendentes, lateral y medial, que en niveles rombencefálicos están separados por una zona ocupada por aferencias del oido interno (Fritzsch, 1988). El lateral coincide con nuestra descripción de las aferencias primarias, si bien podría igualmente contener axones no primarios del PDCS, presente en otros vertebrados (A. Muñoz y cols., 1995b). El medial, formado por fibras gruesas densamente empaquetadas, se extiende rostralmente hasta niveles medios rombencefálicos, donde termina en el lóbulo de la línea lateral. Dicho componente está formado por aferencias de la segunda raíz del complejo de los nervios glosofaríngeo y vago, cuyas ramas descendentes alcanzan niveles espinales, donde ingresan en el DF (Roth y Wake, 1985).

La presencia de aferencias primarias espinales que ascienden en el DF, para terminar en la región del óbex, distintos centros rombencefálicos y en el cerebelo, constituye un carácter común en el cerebro de los vertebrados, aunque existen algunas diferencias respecto a los distintos lugares de destino descritos en los diferentes trabajos (ver Tabla 1 del artículo 2), que quizás reflejen variaciones en cuanto a las aproximaciones experimentales empleadas para su estudio. Así, se ha observado un patrón de organización similar en agnatos (Northcutt y Ebbesson, 1980; Ronan y Northcutt, 1990; Dubuc y cols., 1993), condrictios (Hayle, 1973a; Ebbesson y Hodde, 1981), teleósteos (Oka y cols., 1986; Ronan y Northcutt, 1990), anfibios (Joseph y Whitlock, 1968a, Antal y cols., 1980; Nikundiwe y cols., 1982, M. Muñoz y cols., 1991; A. Muñoz y cols., 1995b), reptiles (Ebbesson, 1967, 1969; Jacobs, 1968; Joseph y Whitlock, 1968b; Pedersen, 1973; Jacobs y Sis, 1980; Kusuma y ten Donkelaar, 1980; Ebbesson y Goodman, 1981; Künzle, 1982; Künzle y Woodson, 1983), aves (Karten, 1963; van den Akker, 1970; Wild, 1985) y mamíferos (Willis y Coggeshall, 1991). La presencia de proyecciones espinales no primarias del PDCS en el DF, se ha demostrado hasta el momento en todos los vertebrados terestres (Rustioni, 1973; Angaut-Petit, 1975a,b; Rustioni y Kaufman, 1977; Bennett y cols., 1984; Giesler y cols., 1984; Funke, 1988; ten Donkelaar y de Boer-van Huizen, 1991; Pritz y Stritzel, 1994; A. Muñoz y cols., 1995b). Sin embargo, su existencia en agnatos, elasmobranquios y teleósteos no ha sido investigada hasta el momento.

Proyecciones incluídas en el funículo dorsolateral (DLF).

En el presente estudio se ha demostrado la existencia en anuros de un sistema de proyecciones, originado en todos los niveles espinales, que asciende a través del funículo dorsolateral. Hemos observado que rostralmente a la aplicación de los diversos trazadores en distintos niveles espinales, dicho sistema inerva los campos espinales dorsal y lateral, y con mayor densidad el núcleo cervical lateral en el nivel cervical superior. Esta última proyección se ha propuesto como el equivalente en anfibios del tracto espinocervical (ver capítulo 5 de la presente memoria). Algunas de las fibras que alcanzan el núcleo cervical lateral, a través del DLF, corresponden a aferencias primarias braquiales que ascienden por el tracto de Lissauer (Joseph y Whitlock, 1968a, Antal y cols., 1980; Nikundiwe y cols., 1982, M. Muñoz y cols., 1991; A. Muñoz y cols., 1995b). A lo largo del rombencéfalo las fibras del DLF se desplazan ventrolateralmente y ascienden inmediatamente

ventrales al tracto descendente del nervio trigémino, organizando proyecciones que alcanzan el polo caudal del núcleo del tracto solitario, y mayoritariamente la formación reticular lateral, y rostralmente el área parabraquial en la región subcerebelosa. Un menor número de fibras penetran en la capa granular del cerebelo, y otras continúan rostralmente para terminar en el polo caudal del núcleo posterodorsal tegmental mesencefálico. En algunas ocasiones se observaron fibras cruzando en la comisura del velo medular anterior. En todos los casos se observó una inervación más densa desde niveles cervicales y torácicos que desde niveles espinales lumbares.

En *Pleurodeles waltl*, en experimentos con aplicaciones de trazadores a nivel cervical, se observó un patrón de marcaje similar al de anuros. Las fibras ascendentes en el DLF organizan, en niveles cervicales superiores de transición con el rombencéfalo, un campo denso de terminales en la región ventrolateral del asta dorsal. Dicho campo terminal ya fue sugerido en *Ambystoma tigrinum* por Herrick (1930). En dicha localización se ha considerado la posible existencia del equivalente en urodelos del núcleo cervical lateral de mamíferos, debido a sus proyecciones al torus semicircularis y al tálamo ventral (ver capítulo 6 de la presente memoria). En experimentos con aplicaciones de BDA en la segunda raíz dorsal espinal se observó que algunas aferencias braquiales ascienden en el tracto de Lissauer, a través del DLF, hasta niveles del núcleo glosofaríngeo y alcanzan a nivel del óbex el núcleo cervical lateral (ver capítulo 6 de la presente memoria). Las fibras no primarias del DLF ascienden a través del rombencéfalo en la sustancia blanca inmediatamente ventral al tracto descendente del nervio trigémino hasta niveles más rostrales, dando a lo largo de todo su recorrido fibras varicosas terminales que penetran hasta la gris periventricular entre los niveles de las raíces del complejo glosofaríngeo-vago y la del trigémino. Rostralmente inervan densamente la región subcerebelosa y en menor medida el tegmento mesencefálico caudal.

La existencia del tracto espinocervical en el DLF, descrito inicialmente en mamíferos (ver Willis y Coggeshall, 1991), es un hecho más común en el cerebro de todos vertebrados de lo que se suponía (ver tabla 2 del artículo 2). Su presencia se ha demostrado recientemente en anfibios (ver capítulo 5 de la presente memoria). Además existen evidencias que sugieren que dicho sistema existe en agnatos (Northcutt y Ebbesson, 1980; Ronan y Northcutt, 1990), condrictios (Hayle, 1973a,b; Ebbesson y Hodde, 1981), teleósteos (Hayle, 1973a,b; Finger, 1981; Ito y cols., 1986), reptiles (Ebbesson, 1967; Künzle y Woodson, 1982), así como en aves (van den Akker; 1970; Funke y Necker, 1986; Funke, 1988; Necker, 1991). Otras proyecciones a través del DLF descritas en anfibios en el presente trabajo incluyen las espinosolitarias, espinoreticulares y espinoparabraquiales que son igualmente comunes en todas las clases de vertebrados (ver Tabla 2 del artículo 2). Por último, las proyecciones espinales que a través del DLF alcanzan el tegmento mesencefálico caudal, tanto en anuros como en urodelos, podrían representar el equivalente de las proyecciones espinales en mamíferos que terminan en la sustancia gris periventricular y en el núcleo cuneiforme (Nijensohn and Kerr, 1975; Zemlan et al., 1984; Björkeland and Boivie, 1984; Hylden et al., 1986; Yezierski, 1988; Bernard et al., 1995).

Proyecciones incluídas en el cuadrante ventral ventrolateral (funículos ventral y ventrolateral) (VQ).

En nuestros experimentos con aplicaciones de diversos trazadores en el asta dorsal de distintos niveles espinales en *Rana perezi* y *Xenopus laevis* hemos observado que las proyecciones espinales ascendentes presentes en el VQ, son bilaterales aunque con predominio contralateral. Dichas proyecciones se originan en neuronas espinales, cuyos axones se dirigen ventromedialmente en el mismo nivel en el que se encuentra el soma neuronal, ingresan en el VQ y ascienden hasta distintos centros supraespinales. El presente estudio de trazado neuronal, demuestra un patrón más amplio en cuanto al número de centros rombencefálicos y mesencefálicos inervados por el VQ que los resultados descritos en trabajos previos, obtenidos mediante técnicas degenerativas (Ebbesson, 1969, 1976; Hayle, 1973a). En niveles rombencefálicos numerosas fibras terminan en los núcleos inferior, medio y superior de la formación reticular medial, mientras que la formación reticular lateral recibe únicamente algunas fibras. Los núcleos motores de los nervios glosofaríngeo y vago, y los del rafe y descendente del nervio trigémino están igualmente inervados. Más rostralmente, las fibras del VQ inervan la región situada entre los núcleos motores de los nervios trigémino y facial, y de manera más dispersa la sustancia gris central rombencefálica y los núcleos dorsal y ventral del nervio octavo. Algunas fibras terminan en la región subcerebelosa y en la capa granular del cerebelo. En niveles ístmicos se observaron fibras marcadas en regiones ventromediales, ventrolaterales y mediales al núcleo de istmo. En el mesencéfalo caudal el VQ inerva los núcleos posterodorsal y posteroventral tegmentales, aunque la mayor parte de las fibras presentes en este nivel, se dirigen dorsalmente para inervar los núcleos laminar, magnocelular, principal y en menor medida el comisural del torus semicircularis, y el techo óptico.

La presencia en el VQ de aferencias espinales (presente estudio), trigeminales (M. Muñoz y cols., 1994, A. Muñoz y cols., 1995b), y de los núcleos de la columna dorsal (Wilczynski, 1981; Wilczynski y Neary, 1986; A. Muñoz y cols., 1994b, 1995b) y cervical lateral (A. Muñoz y cols, 1995b, artículo 3 del capítulo 5 de la presente memoria) permiten la llegada de información somatosensorial al torus semicircularis, y por tanto la existencia de un mapa de representación de la superficie corporal en dicho centro, descrito por Comer and Grobstein (1981c), que interviene en la elaboración de respuestas frente a estímulos tactiles (Comer and Grobstein, 1981a,b). En niveles mesencefálicos rostrales hemos observado fibras en la sustancia gris pretectal y pretoral, en la comisura posterior, así como en las regiones anterodorsal y anteroventral mesencefálicas, donde se han descrito el núcleo rojo y el núcleo del fascículo longitudinal medial.

En estudios morfológicos, basados en técnicas degenerativas (Ebbesson, 1969; Hayle, 1973), no se han descrito hasta el momento proyecciones espinotalámicas directas. Sin embargo, Vesselkin y cols. (1971), en un estudio electrofisiológico en *Rana temporaria* mediante estimulación del nervio ciático, demostraron el procesamiento bilateral de información somatosensorial en toda la extensión rostrocaudal del tálamo dorsal.

En los dos artículos del presente capítulo mediante trazado anterógrado se demuestra la existencia de proyecciones espinotalámicas directas, a través del

sistema VQ, que alcanzan el tálamo dorsal y ventral. En el tálamo dorsal se observó un escaso número de fibras marcadas en los núcleos posterior, central y en menor medida en el anterior. Sin embargo, la mayor densidad de fibras termina en la parte dorsomedial del tubérculo posterior, y en los núcleos ventrolateral (división ventral) y ventromedial del tálamo ventral.

En urodelos Herrick (1936, *Ambystoma tigrinum*) sugirió la inervación espinal de la zona medial del tálamo dorsal. Sin embargo, estudios basados en técnicas de degeneración anterógrada (Nieuwenhuys y Cornelisz, 1971) o en técnicas de trazado con HRP (Wicht y Himstedt, 1988) no han confirmado las consideraciones de Herrick. En el presente estudio, en *Pleurodeles waltl*, se ha demostrado, al igual que en anuros, la presencia de un sistema de fibras ascendentes en el VQ que terminan en la formación reticular rombencefálica, área octavolateral y cerebelo. En el mesencéfalo se observaron fibras terminales marcadas en la sustancia blanca adyacente al torus semicircularis, áreas tegmentales, y en menor número en el techo óptico. En el diencéfalo, la mayoría de las fibras termina en el tálamo ventral, si bien se observó un menor número en las zonas anteroventral y porterodorsal del tálamo dorsal.

No existen datos en la bibliografía sobre la localización y morfología de las neuronas que originan las proyecciones espinales ascendentes en anfibios. En el artículo segundo de este capítulo se presentan resultados correspondientes a experimentos con aplicaciones de BDA en el tálamo ventral, torus semicircularis y núcleo reticular inferior. En todos los casos se observó un mayor número de neuronas contralaterales que ipsilaterales, retrógradamente marcadas en la médula espinal, de acuerdo con los datos de trazado anterógrado anteriormente descritos, procedentes de experimentos con aplicaciones de distintos trazadores en la médula espinal.

En los casos en que se realizaron aplicaciones de BDA en el tálamo se observaron neuronas de diferentes morfologías en niveles cervicales y torácicos, mayoritariamente en el campo dorsal, y en menor número en el lateral y ventral. Las aplicaciones de BDA en el torus semicircularis marcaron células preferentemente en niveles cervicales y en menor grado en segmentos torácicos y lumbares, en las zonas profundas del campo dorsal, y en los campos lateral, ventromedial, ventrolateral y lateral motor. En experimentos con aplicaciones de BDA en el núcleo reticular inferior se obtuvieron neuronas marcadas hasta niveles lumbares superiores, aunque la mayor densidad neuronal se observó en segmentos cervicales, en los campos dorsal (superficial y profundo), lateral, ventromedial y ventrolateral.

En experimentos en *Pleurodeles waltl* con aplicaciones de trazadores retrógrados en el tálamo ventral y en el torus semicircularis, resultaron marcadas neuronas, en su mayoría contralaterales preferentemente en el asta dorsal, y en menor número en la zona intermedia y el asta ventral en niveles espinales cervicales.

La presencia de un sistema de proyecciones ascendentes espinales en el VQ, que en ocasiones se ha denominado lemnisco espinal debido a los trabajos de Herrick (1948) en *Ambystoma tigrinum*, es un caracter común en el cerebro de los vertebrados, si bien existen diferencias en cuanto a los distintos tractos incluídos en dicho sistema en cada grupo (ver tabla 3

del artículo 2). Sin embargo, dichas diferencias, al menos en parte, podrían ser debidas a las distintas metodologías aplicadas en cada estudio. La presencia en el VQ del tracto espinoreticular es común en todos los vertebrados. Sin embargo, el tracto espinocerebeloso ventral parece estar únicamente presente en gnatóstomos. Las proyecciones espinomesencefálicas son propias de todas las clases de vertebrados, en particular terminando en la región intercolicular. Keveter y Willis (1984) basándose en resultados negativos en anamniotas, procedentes de estudios previos de degeneración anterógrada (Hayle, 1973a; Ebbesson, 1969), y de trazado neuronal (Neary and Wilczynski, 1977, 1979), describieron la presencia de proyecciones espinotalámicas como un carácter exclusivo de amniotas, con la excepción de algunos condrictios (Ebbesson y Hodde, 1981) en los que su presencia se consideró un carácter que evolucionó independientemente (Keveter y Willis, 1984). Sin embargo, trabajos posteriores en telecósteos (Murakami e Ito, 1985), anfibios (presente capítulo), y posiblemente en agnatos (Ronan y Nothcutt, 1990), han demostrado proyecciones espinotalámicas en anamniotas, por lo que la presencia de dichas proyecciones directas debe considerarse como un carácter común para todos los vertebrados.

CAPÍTULO 4

Ontogenia del sistema Columna Dorsal-Lemnisco Medial **132**

4.1.- *Early development of dorsal column-medial lemniscal projections in the clawed toad, Xenopus laevis*

4.2.- Comentarios

Early development of dorsal column-medial lemniscal projections in the clawed toad Xenopus laevis

4.1

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ABSTRACT

In *Xenopus laevis* fluorescent dextran amines were applied to study the development of the dorsal column-medial lemniscal projection: rhodamine dextran amine was applied at the mesodiencephalic border to retrogradely label the cells of origin of the medial lemniscus in the dorsal column nucleus (DCN); fluorescein dextran amine to the spinal cord to anterogradely label the primary afferent projections to the DCN. The first mesodiencephalic projections were found at stage 51, i.e. almost immediately after spinal afferent fibers had reached the DCN.

In terrestrial vertebrates, basically two systems of ascending spinal projections are found: (1) a *primary* afferent ascending spinal projection via the dorsal funiculus to the dorsal column nucleus (DCN), giving rise to the medial lemniscal pathway to the thalamus; (2) a *secondary* afferent projection via the lateral funiculus, i.e. the spinal lemniscus, to the reticular formation, mesencephalon and thalamus[25]. Despite rather extensive somatotopically arranged spinal afferents to the anuran dorsal column nucleus[1,9,13,19], only a relatively sparse lemniscal channel projecting to the torus semicircularis in the midbrain, and to thalamic levels, appears to be present[11,24]. In frogs somatosensory information reaches the striatum[22] and the medial pallium[10,14,21] via various thalamic nuclei.

Developmental studies[4,18] indicate that the dorsal column-medial lemniscal projection arises rather early in development. In *Xenopus laevis*, HRP applications at the mesodiencephalic border (see Fig. 1) showed that in late *Xenopus laevis* tadpoles, neurons dorsal and lateral to the solitary tract were labeled, contralateral to the application side, in keeping with data in adult ranid frogs[11]. This perisolitary band includes a dorsal column nucleus and the caudal part of the descending trigeminal nucleus. In such HRP experiments (for details on techniques see ref. 18) the presence of DCN-thalamic projections at least as early as stage 56 could be shown. Similar observations were made in bullfrog (*Rana catesbeiana*) tadpoles[4]: retrograde labeling of DCN cells could be obtained by stage VIII/IX[17], comparable to Nieuwkoop and Faber's[12] stage 54 in *Xenopus laevis*. These data suggest that the projection of dorsal root ganglion (DRG) cells to the DCN precedes the projection of these second-order cells to the thalamus. Data from the opossum[6-8] are consistent with these observations. In the rat, however, a temporal overlap has been reported between the arrival of primary afferent projections to the gracile nucleus[23], and the development of thalamic projections from the DCN[2].

In the present study fluorescent dextran amines, sensitive tracers particularly useful in developmental studies[5], were used to analyze the early development of the DCN-medial lemniscal projection in *Xenopus laevis*. (Tetramethyl)rhodamine dextran amine (RDA; D-1817; Molecular Probes Inc., Eugene, OR, USA) was applied at the mesodiencephalic border or at the torus level in the rostral midbrain to retrogradely label the cells of origin of the medial lemniscus. Contralaterally, fluorescein dextran amine (FDA; D-1820) was applied to the dorsal funiculus or to DRG at cervical levels to characterize that part of the perisolitary band receiving primary spinal afferents. In about 35 *Xenopus laevis* tadpoles ranging from stage 47, i.e. at the time of the appearance of the limb buds, until stage 66[12] (metamorphosis complete), RDA and FDA, recrystallized from distilled water onto sharp tungsten needles, were applied under tricaine methanesulphonate (0.1 mg/ml tap water; MS 222; Sandoz). After survival times of 2-3 days, animals were reanesthetised with an overdose of MS222, and perfused through the heart with 0.1 M phosphate buffer (pH 7.4) followed by a fixative containing 4% paraformaldehyde in phosphate buffer. The brain and (rostral) spinal cord were dissected out, embedded in polyacrylamide[18], left overnight in 15% saccharose in 0.1 M phosphate buffer, and cut on a freezing

microtome at 40 µm, either transversally or horizontally. They were mounted in glycerin-gelatin. The pattern of labeling in representative experiments is shown in Fig. 2.

Following RDA application at the mesodiencephalic border or rostral tegmentum mesencephali (including the torus semicircularis), retrogradely labeled cells were observed in the dorsal column nucleus as early as stage 51 (see Fig. 2A). The localization of this neuron population coincides with the place of arborization of ascending collaterals of spinal primary afferents (PAFs; Fig. 2A, arrow). FDA-labeled PAFs were found ascending through the dorsal funiculus and along Lissauer's tract. At the level of the dorsal column nucleus the PAFs give off collaterals that pass ventrally into the grey matter where the RDA labeled cells were found (Fig. 2A). The dendrites of these cells extend laterally and dorsally to the area of FDA labeling. A few laterally situated neurons extend their dendrites dorsomedially. The axons of the DCN neurons could be traced ventromedially to cross the midline towards the contralateral ventral ascending pathway in the brainstem. In stage 51 tadpoles, only a few labeled cells were found. The number of labeled DCN-neurons increased from stage 51 to 55 (e.g. Fig. 2B). From stage 55 on a more or less adult pattern of rostrally projecting DCN cells could be observed (see Fig. 2C,D).

By applying FDA to the dorsal funiculus, in general all fibers passing rostrally at the level of application, mostly the third spinal segment (brachial enlargement), were labeled. In all of the stages studied i.e. from stage 47 until 66, labeled PAFs could be observed (Fig. 2), but a progressive increase of the projections in the youngest stages studied (47-52) was observed. In a previous study[18] it was shown that around stage 48 projections from DRG cells reach the level of the developing DCN, but only from non-limb bud-innervating DRG. Limb bud innervating ganglia give rise to ascending collaterals definitively later in development. At stage 52 a few ascending branches extended into the brain stem[20]. By stage 53 all spinal ganglia including lumbar DRG send ascending collaterals to the brain stem[18]. In addition to the anterogradely labeled PAFs, a few retrogradely labeled neurons were found ipsilaterally in the DCN, at least as early as stage 51. The number of caudally projecting DCN cells increased in later stages.

The present data show that the anuran dorsal column-medial lemniscal system arises earlier than previously thought[4,18]. The first mesodiencephalic projections from the dorsal column nucleus were found at stage 51, i.e. not much later than projections of DRG cells reach the area of the DCN. It should be noted, however, that the first spinal projections reaching the developing dorsal column nucleus arise from thoracic DRG cells[3,15,16,18]. During development, primary sensory neurons innervate peripheral targets and *then* form central connections appropriate to these targets[15,16,20]. The projection of thoracic DRG cells to the DCN precedes the projection of these second-order cells to the thalamus. It seems likely that the projections of cervical and lumbar DRG cells to the DCN also precede the projection of related second-order cells giving rise to the medial lemniscus. Current research is focussed on the application of the sensitive tracer, biotylinated dextran amine, to dorsal root ganglia, in combination

with the application of this tracer at the mesodiencephalic border. In this way it can be established which DRG cells innervate particularly DCN neurons.

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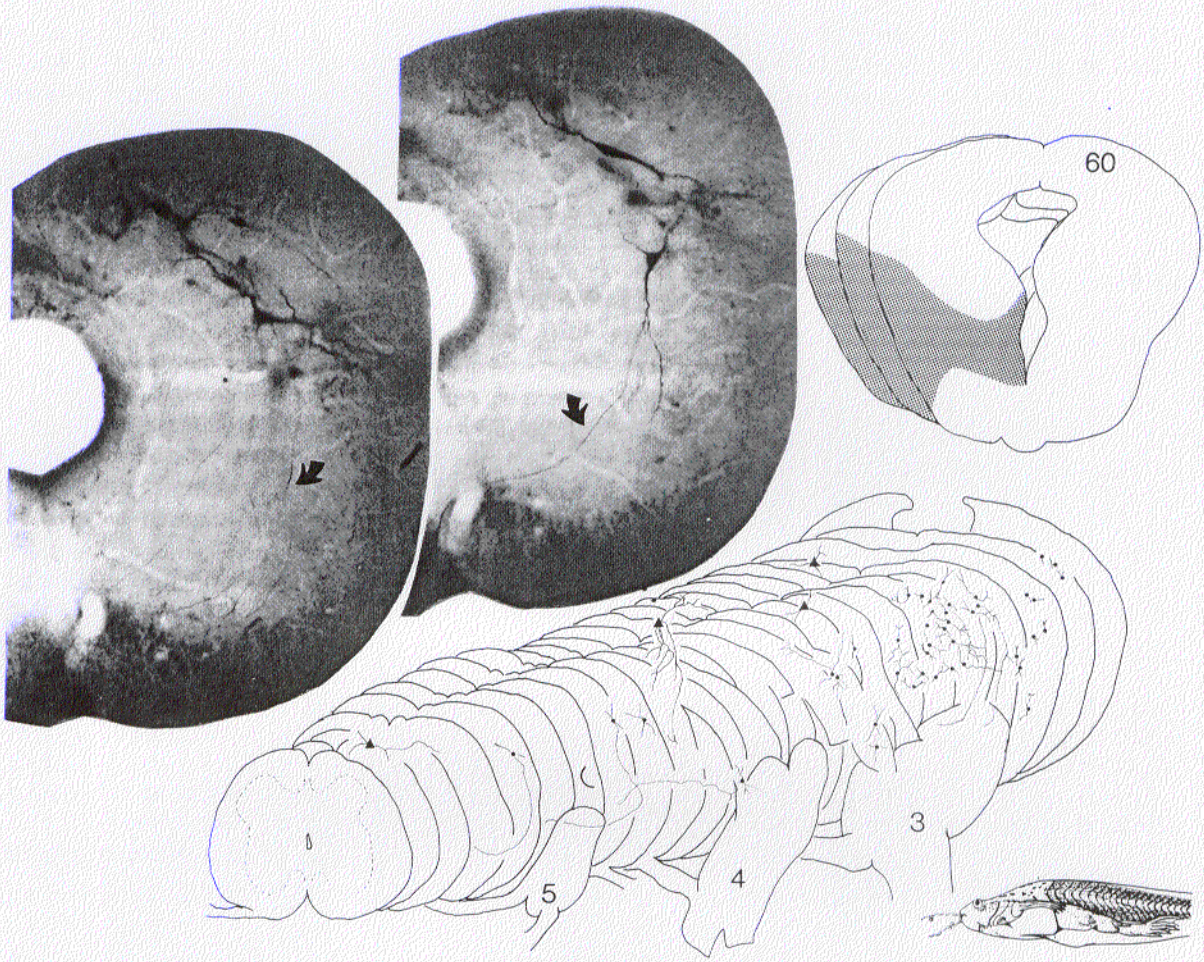

Figure 1: Reconstruction of the caudal brain stem and rostral spinal cord in a late *Xenopus laevis* tadpole (stage 60). HRP was applied at the mesodiencephalic border. Small dots indicate the labeled cells (with dendrites), contralateral to the application side of the tracer; triangles indicate a few ipsilaterally found neurons; DRG 3-5 are shown. Photomicrographs show examples of the labeling of the perisolitary band, modified by the DAB-staining techniques[18] (magnification x 100). Arrows indicate labeled medial lemniscal fibers.

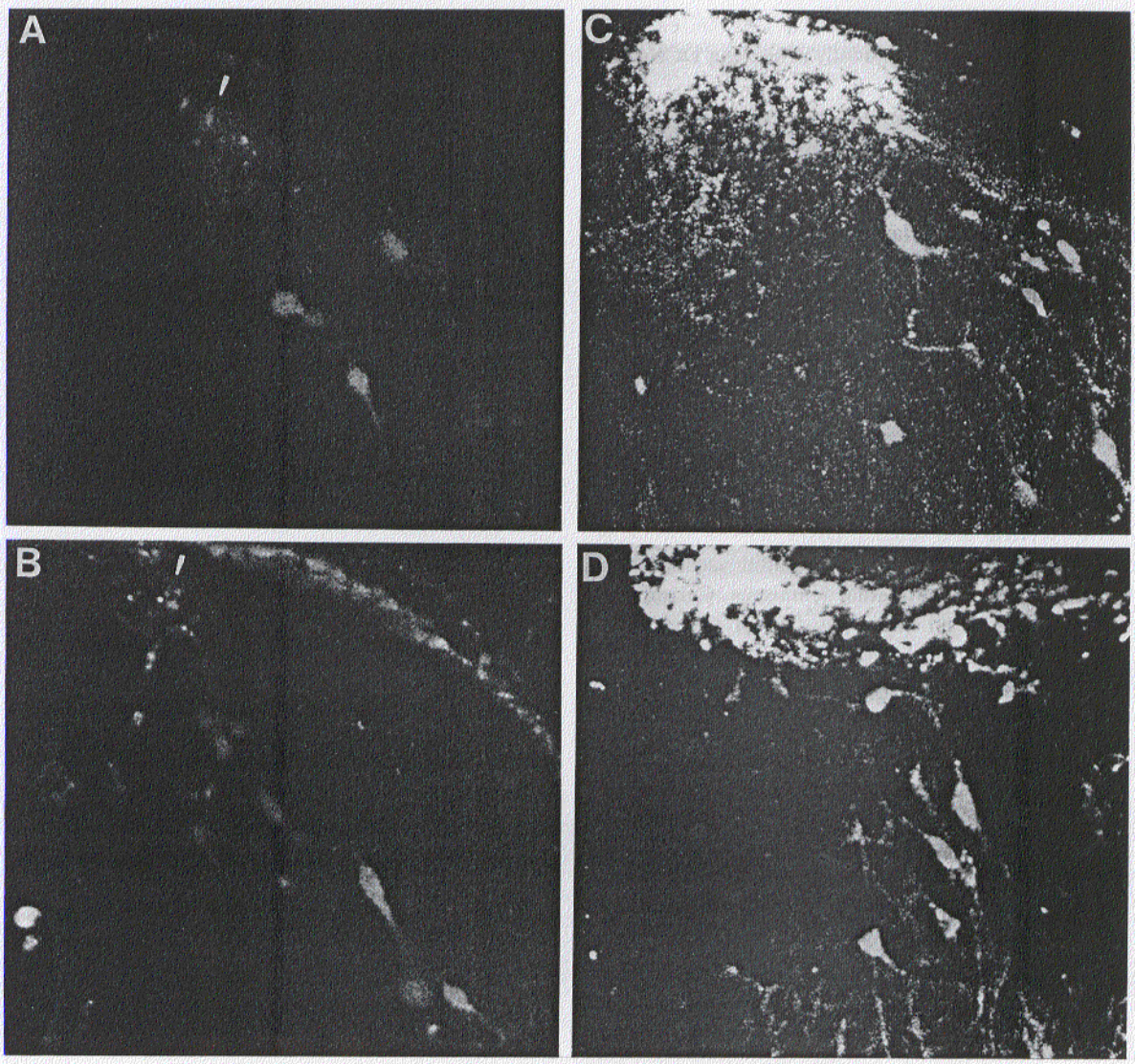

Figure 2: Transverse sections at the obex level of *Xenopus laevis*, showing RDA labeled neurons in the developing dorsal column nucleus. A: in a stage 51 tadpole (magnification x400). B: in a stage 53 tadpole (magnification x550). Superposed pictures A and B were taken with a Zeiss fluorescence microscope with appropriate filter combinations. C,D: in a stage 61 tadpole (magnification x215), confocal microscopy (Bio-Rad Lasersharp MRC-500). Arrows indicate the FDA labeled spinal primary afferents.

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Early development of dorsal column-medial lemniscal projections in the clawed toad Xenopus laevis

COMENTARIOS

4.2

En vertebrados terrestres el sistema columna dorsal-lemnisco medial está formado por las proyecciones aferentes espinales, primarias y no primarias, que alcanzan los núcleos de la columna dorsal a través del funículo dorsal, y por las proyecciones eferentes de éstos que constituyen el lemnisco medial, el cual asciende contralateralmente hasta el mesencéfalo y el tálamo (Willis y Coggeshall, 1991).

En anfibios existe un sistema de aferencias espinales primarias, somatotópicamente organizado, que alcanza el núcleo de la columna dorsal (DCN) (Antal y cols., 1980; Nikundiwe y cols., 1982; Urbán y Székely, 1982). Sin embargo, parece existir un canal lemniscal poco desarrollado, que proyecta al torus semicircularis en el mesencéfalo, y a niveles talámicos (Neary y Wilczynski, 1977; Wilczynski y Neary, 1986).

Estudios sobre el desarrollo larvario de las proyecciones espinales ascendentes (Forehand y Farel, 1982; ten Donkelaar y de Boer-van Huizen, 1991) indican que la proyección columna dorsal-lemnisco medial aparece en estadios tempranos del desarrollo. ten Donkelaar y de Boer-van Huizen (1991) demostraron que las aferencias primarias espinales alcanzan la región del óbex entre los estadios 48 y 53 (Nieuwkoop y Faber, 1967) en *Xenopus laevis*. Forehand y Farel (1982) en experimentos con aplicaciones talámicas de HRP en distintos estadios del desarrollo en *Rana catesbeiana*, obtuvieron marcaje retrógrado de neuronas del DCN en animales del estadio VIII/IX (Taylor y Kollros, 1946), comparable al estadio 54 de Nieuwkoop y Faber (1967) en *Xenopus laevis*. Estos datos sugieren que el desarrollo de la proyección de las neuronas ganglionares de las raíces dorsales al DCN, precede al de la proyección talámica de este núcleo.

En mamíferos, los datos obtenidos en la zarigüella (Johnson y cols., 1972; Martin y cols., 1983, 1987) coinciden con dichas observaciones. En la rata, sin embargo, se ha descrito un solapamiento temporal entre la llegada de aferencias primarias al núcleo *gracilis*, (Wessels y cols., 1991) y el desarrollo de las proyecciones talámicas desde los núcleos de la columna dorsal (Asanuma y cols., 1988).

En el presente capítulo se ha estudiado el desarrollo de las proyecciones columna dorsal-lemnisco medial en *Xenopus laevis*, mediante la aplicación en animales entre los estadios 47 y 66 del desarrollo, de dextrano aminas combinadas con rodamina (RDA) en el torus semicircularis y en la zona mesodiencefálica, con objeto de marcar en el DCN retrógradamente las células de origen del lemnisco medial. En los mismos experimentos se aplicó contralateralmente dextrano aminas combinadas con fluoresceína (FDA) en la médula espinal o en los ganglios de la raíces dorsales, con objeto de marcar las proyecciones aferentes al DCN.

Las aplicaciones de RDA en la zona mesodiencefálica o en el torus semicircularis marcaron neuronas en el DCN desde el estadio 51, en el que observa un escaso número de células, que se incrementa progresivamente entre los estadios 51 y 55. A partir del estadio 55 se establece un patrón, más o menos semejante al del adulto, de neuronas del DCN de proyección rostral. La localización de esta población neuronal, coincide con el sitio de arborización de las fibras primarias espinales, marcadas anterógradamente con FDA desde los

ganglios dorsales o la médula espinal, que ascienden a través del funículo dorsal y del tracto de Lissauer en el funículo dorsolateral. Asimismo se observó que las dendritas de las neuronas del DCN, marcadas con RDA, se extienden dorsal y lateralmente hacia el área ocupada por las fibras marcadas con FDA. En todos los estadios estudiados se pudieron observar aferencias primarias espinales marcadas, pero se produce un incremento progresivo del número de fibras entre los estadios 47 y 52.

En un estudio previo ten Donkelaar y de Boer-van Huizen (1991) demostraron que hacia el estadio 48, las proyecciones desde neuronas de los ganglios de la raíces dorsales alcanzan el DCN, pero únicamente desde los ganglios torácicos que no inervan los primordios de las extremidades. Los ganglios que inervan las extremidades originan ramas ascendentes en estadios más avanzados. En el estadio 52 algunas fibras alcanzan el tronco cerebral (van Mier y ten Donkelaar, 1988), y en el estadio 53 todos los ganglios espinales, incluyendo los lumbares, envían colaterales ascendentes al tronco cerebral (ten Donkelaar y de Boer-van Huizen, 1991).

El presente estudio demuestra que el sistema columna dorsal-lemnisco medial en anuros, se origina en estadios anteriores a lo que se había descrito previamente (Forehand y Farel, 1982; ten Donkelaar y de Boer-van Huizen, 1991). Nuestros resultados indican que la proyección de las neuronas ganglionares torácicas al DCN, precede al desarrollo de la proyección talámica de dicho núcleo. Las primeras proyecciones mesodiencefálicas desde el DCN se originan en el estadio 51, poco después de que este núcleo reciba las primeras aferencias espinales, desde células ganglionares torácicas (estadio 47) (Clarke y cols., 1986; Smith y Frank, 1988a, b; ten Donkelaar y de Boer-van Huizen, 1991). Durante la ontogenia, las neuronas sensoriales primarias inervan primero las dianas periféricas y posteriormente establecen conexiones centrales apropiadas a dichas dianas (Smith y Frank, 1988a, b; van Mier and ten Donkelaar, 1988). ten Donkelaar y de Boer-van Huizen (1991) observaron que los axones de las neuronas de los ganglios cervicales y lumbares, alcanzan el área del DCN en los estadios 52-53. Nuestros resultados demuestran que durante los estadios 51 a 55 se produce un incremento en el número de células del DCN cuyos axones llegan al borde mesodiencefálico. Por lo tanto, parece probable que el desarrollo de las proyecciones primarias al DCN desde los ganglios cervicales y lumbares (estadios 52-53), preceda igualmente al aumento de la proyección lemniscal desde las neuronas del DCN, relacionadas con dichas aferencias, pudiendo existir así, en la ontogenia de las proyecciones somatosensoriales, un patrón de determinación periférico-central del sistema columna dorsal-lemnisco medial.

CAPÍTULO 5

El Lemnisco Medial en anuros 143

5.1.- *The dorsal column-medial lemniscal projections of anuran amphibians*

5.2.- *The anuran dorsal column nucleus: Organization, immunohistochemical characterization, and fiber connections in Rana perezi and Xenopus laevis*

5.3.- *Evidence for an anuran homologue of the mammalian spinocervicothalamic system: An in vitro tract tracing study in Xenopus laevis*

5.4.- Comentarios

The dorsal column-medial lemniscal projection of anuran amphibians

5.1

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ABSTRACT

The efferent connections of the dorsal column nucleus (DCN) of the anuran amphibians *Rana ridibunda* and *Xenopus laevis* have been studied by means of bidirectionally transported tracres. Efferent projections from the DCN innervate the spinal cord, tegmentum of the brain stem, cerebellum, torus semicircularis and thalamus. The pattern of connectivity of the anuran DCN is largely comparable to that of amniotic vertebrates although some peculiarities are found.

The dorsal column-medial lemniscal system (DC-ML) i.e. the primary afferent spinal projection via the dorsal funiculus to the dorsal column nucleus (DCN), which gives rise to the medial lemniscal projection to the thalamus, represents a main ascending spinal projection in vertebrates (see Willis & Coggeshal, 1991). The existence of such a projection in anurans has been a much debated question and the connectivity of the DCN in amphibians is only partially known. The termination of the primary afferents to the DCN shows a somatotopic organization in relation to the rostrocaudal spinal level of their origin (Antal et al., 1980; Nikundiwe et al., 1982; Urbán & Székely, 1982). An efferent DCN projection to the contralateral thalamus has been obseved in ranid frogs in physiologycal stimulation (Vesselkin et al., 1971; Urbán & Székely, 1982) and tracing studies (Neary & Wilczynski, 1977). Also a medial lemniscal innervation of the torus semicircularis (Comer & Grobstein, 1981; Wilczynski, 1981; Wilczynski & Neary, 1986; Muñoz et al. 1993) has been demonstrated, and retrogradely labeled cells were observed in the DCN after cerebellar (González et al., 1984; Grover & Grüsser-Cornehls, 1984) and tectal tracer applications (Zittlau et al, 1988). In the pressent study the main ascending projections of the anuran DCN will b discussed.

In 15 adults of *Rana ridibunda* and 10 young adults of *Xenopus laevis* under anesthesia with MS222, HRP crystals or biotinylated dextran amine (BDA, Mollecular Probes) recrystallized from destilled water onto sharp tungsten needles were applied to the DCN area, or BDA or rhodamine dextran amine (RDA, Lollecular Probes) were applied as crystals or iontophoretically injected into the thalamus, torus semicircularis and cerebellum. After 2-10 days of survival time the animals were reanesthetized and perfused through the heart with saline followed by 4% paraformaldehide (2.5% paraformaldehide with 2% glutaraldehide for HRP experiments). The brain and spinal cord were then removed and stored overnight in a 30% saccharose solution. Frontal, 40 µm thick sections were cut on a frezzing microtome. HRP material was reacted with DAB as chromogen, usually intensified with nickel. To visualize BDA, a Vectastain ABC elite kit (Vector) was used, followed by the HRP reaction with DAB. Although the iontophoretic injections of the tracers were rather small, particularly those with BDA, it was always hardly possible to restrict the tracer to the area of the DCN. Medially located injections often involved partially the nucleus of the solitary tract, whereas more lateral injections included part of the nucleus of the descending trigeminal tract. Thus, solely with this type of tracer application it is not possible to fully discriminate the connections of the DCN. Therefore, subsequent retrograde tracing experiments were used to confirm the projections from the DCN. Since the results in both species are largely comparable we will describe the general pattern of the connections of the DCN. Only when species differences are found will then be mentioned separately.

Small unilateral applications of HRP or BDA that involved the DCN labeled anterogradely a distinct contralateral ascending system from the DCN, i.e. the medial lemniscus (Fig.1). Its axons could be traced from the injection site and course ventrally and medially, cross to the contralateral side beneath the central canal, and then turn rostralwards into the ventral tegmentum. As the medial lemniscus ascends in the rhombencephalon, smoothly swings to more

dorsolateral positions. All through the medulla, the medial lemniscus gives off thin fibers to various parts of the reticular formation. A few fibers course dorsally into the octavolateral area and some enter the granular layer of the cerebellum (Fig.1E). At caudal mesencephalic levels the fibers turn dorsally along the lateral aspect of the midbrain and most of them bend medially where they terminate in the torus semicircularis (Fig. 1D). The principal, magnocellular and commissural nuclei receive only a sparse DCN projection but the laminar nucleus is densely innervated, particularly its lateral portion. A few fibers cross to the contralateral commissural and principal nuclei of the torus semicircularis. A significant difference between the two species studied is that while in *R. ridibunda* DCN efferent fibers do not reach the tectum, in *X. laevis* intermediate and deep layers are innervated all through the mediolateral tectal extent by collaterals of the toral innervating medial lemniscal fibers. In both species, at more rostral mesencephalic levels (Fig. 1C), the anterodorsal and anteroventral tegmental nuclei including the red nucleus and the nucleus of the fasciculus longitudinalis medialis are innervated by labeled fibers, predominantly contralateral to the DCN injected. At meso-diencephalic transition levels scattered labeled fibers distribute in the pretoral gray. Beyond the midbrain, both dorsal and the ventral thalamic areas are innervated by DCN efferent fibers. The ventral part of the posterior and central dorsal thalamic nuclei are reached by a few thin, varicose fibers whereas the ventromedial thalamic nucleus and the nucleus of the posterior tubercle are far more densely innervated (Fig. 1A,B). No labeling was found more rostrally in the anterior diencephalon or the telencephalon in any of the cases.

Extralemniscal ipsilateral projections ascending all through the rhombencephalon up to the cerebellum were observed. In order to confirm the putative ascending projections of the DCN, HRP, BDA, or RDA injections in both anuran species were placed into the thalamus, torus semicircularis and the cerebellar region. In experiments that affected both the dorsal and the ventral thalamus bilaterally, but mostly contralaterally, irregular large and small round cells with dorsally and ventrolaterally directed processes were observed in the DCN. Their axons could be followed to the contralateral medial lemniscus. When the injection sites were restricted to the torus semicircularis, dorsomedial and lareroventral components of retrogradelly labeled cells were observed in the contralateral DCN. A few ipsilateral cells were also present. The dorsomedial component contains large and small cells with processes directed dorsally, whereas the large bipolar neurons of the lateroventral component posses several processes directed into the dorsal but mainly dorsolateral funiculus. In experiments with applications into the cerebellum, bilaterally but mostly ipsilaterally, retrogradelly labeled cells were seen in the DCN. Their axons seem to course together with the ascending primary afferents from the dorsal roots up to the cerebellum.

As in other vertebrates the dorsal column-medial lemnical projection relays ascendig spinal somatosensory information from the spinal ganglionar cells to different supraspinal targets. Together with the direct spinothalamic projection, recently described (Muñoz et al., 1994), the medial lemniscal innervation of the central and posterior nuclei of the dorsal thalamus represents the anatomical basis of a direct somatosensorial projection, already demonstrated

physiologically (Vesselkin et al., 71). In the ventral thalamus, primaryly the ventromedial nucleus, and the posterior tuberculum are the main diencephalic targets of the medial lemniscal fibers as well as those of the spinal lemniscus (Muñoz et al., 1994). A main component of the anuran medial lemnicus innervates various parts of the torus semicircularis. Our experiments pointed to the lateral aspect of the laminar nucleus as the most densely innervated part of the torus although the principal, magnocellular and in lesser extent the commissural nuclei also receive medial lemniscal innervation. Two different neuronal components in the DCN, dorsomedial and latroventral, were observed to give rise to this projection. The differences in the medial lemniscal innervation of the optic tectum in the species studied agree with previous data in wich DCN cells were descrived after tectal HRP applications in *Xenopus laevis* (Zittlau et al., 1988) but not in *Rana* (Wilczynski & Northcutt, 1977; Hofmann et al., 1990; Masino and Grobstein, 1990). Labeled varicose fibers were observed ipsilaterally at the granular layer of the cerebellum after DCN tracer application. This projection is corroborated by retrograde labeling from cerebellar regions and these results are in line with previous tracing studies both in *Xenopus* (González et al., 1984) and *Rana* (Grover & Grüsser Cornels, 1984).

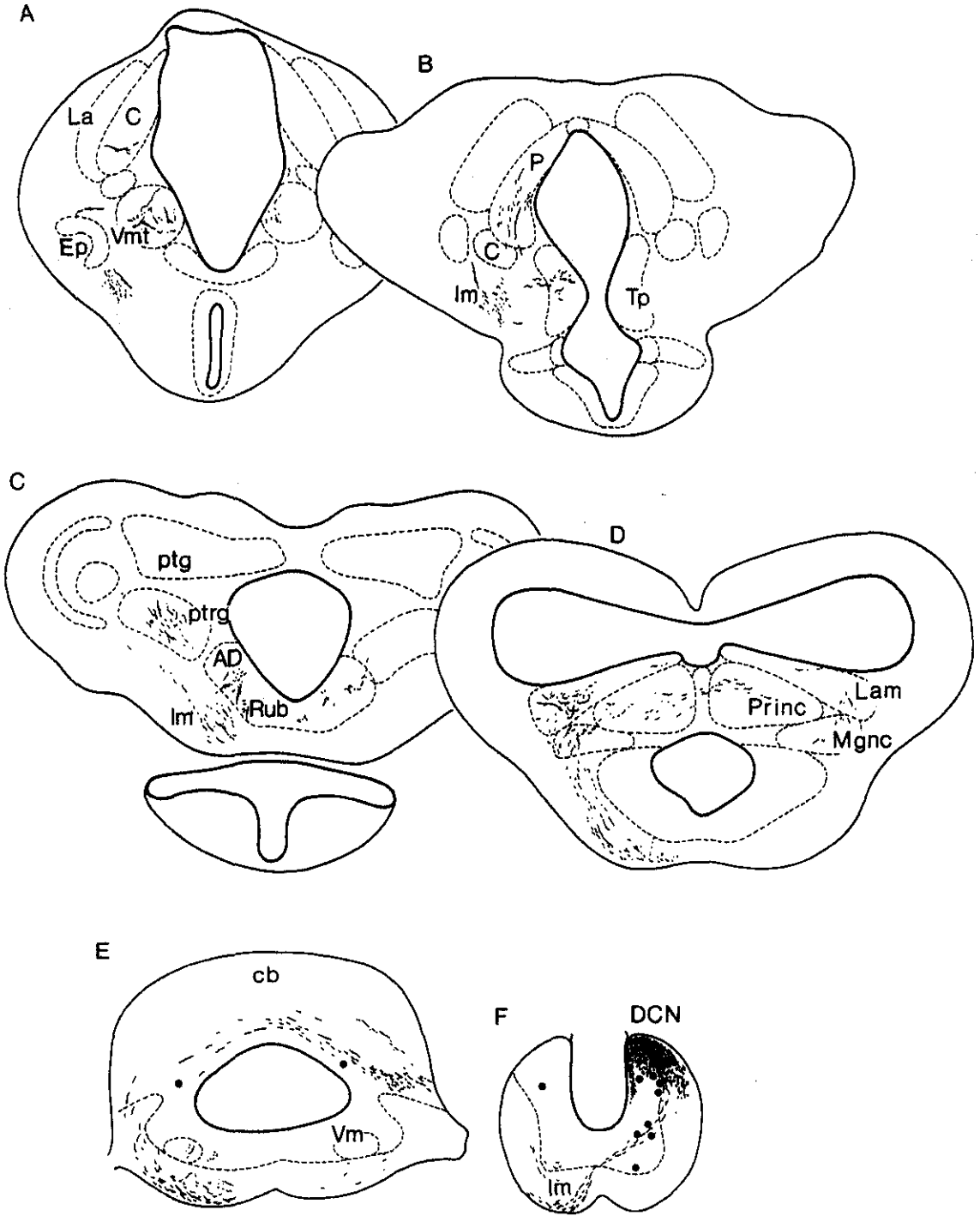

Figure 1: Schematic drawings of transverse sections through the brain of *Rana ridibunda* showing the medial lemniscal targets at diencephalic (A-B), mesencephalic (C-D) and cerebellar (E) levels after a BDA application into the DCN area (F). Abbreviations: AD: anterodorsal tegmental nucleus; C, La, P, Vmt: central, lateral anterior, posterior and ventromedial thalamic nucleus; cb: cerebellum; DCN: dorsal column nucleus; Ep: posterior entopudencular nucleus; Lam, Mgnc, Princ: laminar, magnocellular and principal nucleus of the torus semicircularis; lm: medial lemniscus; prtg: pretoral gray; ptg: pretectal gray; Rub: red nucleus; Tp: nucleus of posterior tubercle; Vm: trigeminal motor nucleus.

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The anuran dorsal column nucleus: Organization, immunohistochemical characterization, and fiber connections in Rana perezi and Xenopus laevis

5.2

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ABSTRACT

As part of a research programme on the evolution of somatosensory systems in vertebrates, the dorsal column nucleus (DCN) was studied with (immuno)histochemical and tract-tracing techniques in anurans (the large green frog, *Rana perezi*, and the clawed toad, *Xenopus laevis*). The anuran DCN contains some nicotinamide adenine dinucleotide phosphate diaphorase-positive neurons, very little calbindin D-28k, and a distinct parvalbumin-positive cell population. The anuran DCN is innervated by primary and non-primary spinal afferents, by primary afferents from the Vth, VIIth, IXth and Xth cranial nerves, by serotonin-immunoreactive fibers, and by peptidergic fibers. Non-primary DCN afferents from the spinal cord appear to arise throughout the spinal cord, but particularly from the ipsilateral dorsal grey. The present study focused on the efferent connections

of the DCN, more in particular the targets of the medial lemniscus. The medial lemniscus could be traced throughout the brainstem, and into the diencephalon. Along its course, the medial lemniscus gives off collaterals to various parts of the reticular formation, to the octavolateral area, and to the granular layer of the cerebellum. At mesencephalic levels, the medial lemniscus innervates the lateral part of the torus semicircularis as well as various tegmental nuclei. A striking difference between the two species studied is that while in *Rana perezi* medial lemniscal fibers do not reach the tectum mesencephali, in *Xenopus laevis*, intermediate and deep tectal layers are innervated. Beyond the midbrain, both dorsal and ventral thalamic areas are innervated by the medial lemniscus. The present study shows that the anuran 'lemniscal pathway' is basically similar to that of amniotes.

INTRODUCTION

In terrestrial vertebrates, two basic systems of ascending spinal projections are found (see Willis and Coggeshall, 1991): 1) a *primary* afferent ascending spinal projection via the dorsal funiculus to the dorsal column nucleus, giving rise to the medial lemniscal pathway to the thalamus, and 2) a *secondary* afferent projection via the lateral funiculus – i.e., the spinal lemniscus – to the reticular formation, mesencephalon and thalamus.

In anurans, anterograde degeneration studies (e.g., Ebbesson, 1969, 1976; Hayle, 1973a,b) did not demonstrate a spinothalamic tract, and the existence of a dorsal column-medial lemniscal system remained a much debated question until the early 1980's. A recent anterograde tracer study showed a distinct direct spinothalamic projection in anurans (A. Muñoz et al., 1994). The anuran dorsal column nucleus (DCN) is somatotopically arranged in such a fashion that its medial ('gracile') compartment is innervated by dorsal root fibers from lumbar and thoracic segments, whereas those of the cervical enlargement project to the lateral ('cuneate') compartment (Antal et al., 1980; Nikundiwe et al., 1982; Jhaveri and Frank, 1983). In *Xenopus laevis*, also, a non-primary afferent projection to the DCN or postsynaptic dorsal column system was demonstrated (ten Donkelaar and de Boer-van Huizen, 1991). In ranid frogs, Vesselkin and co-workers (Vesselkin et al., 1971; Vesselkin and Kovacevic, 1973), Silvey et al. (1974), as well as Neary and Wilczynski (1977) described a contralateral projection of the DCN (or perisolitary band) to thalamic nuclei. More recent cobalt labeling studies in *Rana esculenta* (Antal et al., 1980; Urbán and Székely, 1982), horseradish peroxidae (HRP) tracing in *Rana perezi* (M. Muñoz et al., 1991) and in *Xenopus laevis* (ten Donkelaar and de Boer-van Huizen, 1991; A. Muñoz et al., 1993) suggest that the amphibian DCN-medial lemniscal system in fact might closely resemble the system found in amniotes. With electrophysiological techniques, Urbán and Székely (1982) showed a rather extensive, contralateral thalamic somatosensory projection following stimulation of either the 2nd dorsal root, the dorsal funiculus or the dorsal column nucleus. The anuran medial lemniscus also innervates the lateral part of the torus semicircularis (Comer and Grobstein, 1981; Wilczynski, 1981; Neary and Wilczynski, 1986).

The present study is part of a research programme on the evolution of somatosensory systems in vertebrates. The development, chemical neuroanatomy and circuitry of somatosensory systems is being studied in amphibians: urodeles as well as anurans. In the present study, the connectivity of the

dorsal column nucleus in two anuran amphibians, the Spanish green frog, *Rana perezi* (formerly *R. ridibunda*) and the clawed toad, *Xenopus laevis* was analyzed, using mainly HRP and biotinylated dextran amine (BDA) tracing techniques. Little is known about the chemical neuroanatomy of the anuran DCN. The mammalian cuneate and gracile nuclei are characterized by the presence of GABAergic (inter)neurons, and are innervated by substance P-positive and many other peptidergic fibers (see Rustioni and Weinberg, 1989, for review). Recent studies (e.g., Celio, 1990; Rausell and Jones, 1991a,b; Rausell et al., 1992; Menétrey et al., 1992a,b; Maslany et al., 1992; Ren and Ruda, 1994) also showed a certain preferential distribution of calcium-binding proteins like calbindin D-28k and parvalbumin for somatosensory structures including the dorsal column nuclei. Nitric oxide synthase (NOS) possibly marks a population of local circuit neurons within the DCN (Valtschanoff et al., 1993). No such data are available for anurans. Therefore, the existence of different cell populations within the anuran dorsal column nucleus was studied using nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry (NADPH diaphorase being a marker for nitric oxide synthase), calbindin D-28k, parvalbumin, GABA and glycine immunohistochemistry. Additionally, data on the serotonergic and peptidergic innervation of the DCN will be discussed. It will be shown that the anuran dorsal column–medial lemniscus system is basically similar to that of amniotes.

MATERIALS AND TECHNIQUES

The animals (60 adult specimens of *Rana perezi* and 45 young adult *Xenopus laevis*) were obtained from laboratory stock of the Department of Cell Biology, University Complutense of Madrid *(R. perezi)*, and the Department of Animal Physiology, University of Nijmegen *(X. laevis)*. For a cytoarchitectonic analysis of the obex region, Nissl (cresylecht violet)-stained series of both anurans were available, cut either transversally, horizontally or sagittally at a thickness of 20 µm. Adjacent sections were stained with silver proteinate, either according to Bodian's (1936) or according to Klüver and Barrera's (1953) technique. The histochemical, immunohistochemical and tract-tracing techniques used in this study are discussed below. The nomenclature used is based on studies by Opdam et al. (1976) and Nikundiwe and Nieuwenhuys (1983) on the brain stem, and by Neary and Northcutt (1983) on the anuran diencephalon.

NADPH-diaphorase histochemistry

Four adult frogs *(Rana perezi)* were anesthetized in a 0.3% solution of tricaine methanesulphonate (MS222, Sandoz), and subsequently perfused transcardially with a 0.9% saline solution followed by a fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were taken out and further fixed in the same fixative for six to eight hours at room temperature. They were subsequently immersed in a 30% sucrose phosphate buffer solution at 4¤C, embedded in a 15% gelatin and 30% sucrose solution, and stored for five hours in a 4% formaldehyde solution at room temperature. On a freezing microtome, 30 or 40 µm frontal sections were cut and collected in phosphate buffer. Free-floating sections were incubated in a medium containing 1mM ß-NADPH, 0.8mM nitroblue tetrazolium and 0.06% Triton X-100 in

0.1M phosphate buffer (pH 7.6) at 37¤C for one to two hours. After incubation, the sections were thoroughly rinsed in phosphate buffer, mounted on gelatin-coated glass slides, and, after drying overnight, coverslipped. Selected sections were counterstained with 1% cresyl violet. In two cases, the sections were also processed for tyrosine hydroxylase immunohistochemistry after rinsing.

Immunohistochemical procedures

For the immunohistochemical procedures used, animals were anesthetized with an overdose of MS222, and transcardially perfused with saline followed by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were removed and postfixed for 4-7 hours in the same fixative, embedded in 15% gelatin with 30% sucrose *(Rana perezi)* or in polyacrylamide *(Xenopus laevis)*. Brains were cut frontally on a freezing microtome or on a vibratome at 40 µm, and the sections were collected in a Tris-saline (TBS) buffer (0.05M, pH 7.6). All antibodies were diluted in 0.1% normal serum of the species in which the secondary antibody was raised, in TBS with 0.1% Triton X-100 (Sigma). The sections were preincubated for 1-2 hours in TBS-containing 3% normal serum and 0.1% Triton X-100 and subsequently incubated in the primary antibody-containing solution for 24-36 hours at 4¤C. Controls for the immunohistochemistry experiments included: 1) staining some selected sections with pre-immune mouse serum (1:1,000 for tyrosine hydroxylase, calbindin D-28k and parvalbumin immunohistochemistry), or with rabbit serum (1:500 for glycine; 1:1,000 for GABA, neuropeptide Y and serotonin; 1:2,000 for calcitonin-gene related peptide, substance P, and Leu-enkephalin immunohistochemistry) instead of the primary antibody (e.g., Fig. 5C), and 2) controls in which primary antibody, secondary antibody or the peroxidase-antiperoxidase complex was omitted. As an additional control for the specificity of the labeling of calcium-binding proteins, some sections were stained using antibodies that had been pre-absorbed in an excess of parvalbumin or calbindin. These procedures revealed a light, diffuse background. No stained cells or fibers were found in any of the cases. The sections were processed according to the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979) in a series of incubations with the following antisera:

1) *tyrosine hydroxylase (TH) immunohistochemistry* (8 cases): a) mouse anti-TH (Incstar), diluted 1:1,000, for 24-72 hours; b) goat antimouse (Nordic), diluted 1:100, for 3-5 hours, and c) rat peroxidase-antiperoxidase (PAP) complex (Incstar), diluted 1:500, for 2 hours;

2) *calbindin D-28k and parvalbumin immunohistochemistry* (12 cases): a) mouse anti-calbindin D-28k (Sigma) and mouse anti-parvalbumin (Sigma), diluted 1:1,000, for 24-72 hours; b) goat antimouse (Nordic), diluted 1:100, for 3-5 hours, and c) PAP, diluted 1:500, for 2 hours;

3) *GABA immunohistochemistry* (8 cases) after colchicine (Sigma) injections (3 µl, containing 20 µg/µl) into the fourth ventricle of deeply anesthetized animals, and perfusion after survival times of 5-12 hours, the following procedure was used: a) rabbit anti-GABA (Sigma), diluted 1:1,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) PAP-rabbit (Dakopatts), diluted 1:600, for 2 hours;

4) *glycine immunohistochemistry* (5 cases): a) rabbit anti-glycine (Chemicon), diluted 1:200-1:500, for 24 hours; b) goat anti-rabbit (Sigma), diluted

1:50-1:200, and c) rabbit PAP-complex (Dakopatts), diluted 1:600, for 2 hours;

5) *calcitonin-gene related peptide (CGRP) immunohistochemistry* (5 cases): a) rabbit anti-CGRP (Amersham), diluted 1:2,000, for 12-24 hours; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for 2 hours;

6) *substance P (SP) (5 cases) and Leu-enkephalin (L-Enk) immunohistochemistry* (4 cases): a) rabbit anti-SP or rabbit anti-L-Enk (CRB), diluted 1:2,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for 2 hours;

7) *neuropeptide Y immunohistochemistry* (4 cases): a) rabbit anti-NPY serum (gift from Dr. J.D. Mikkelsen), diluted 1:1,000, for 36 hours; b) swine anti-rabbit (Nordic), diluted 1:50, for 1 hour, and c) rabbit PAP complex (Dakopatts), diluted 1:800, for 1 hour;

8) *serotonin (5-HT) immunohistochemistry* (5 cases): a) rabbit anti 5-HT (gift from Dr. H.W.M. Steinbusch), diluted 1:1,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for 2 hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for 2 hours.

In all cases, after rinsing, the sections were incubated with 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in phosphate buffer, for 10-15 minutes. After another rinsing, the sections were mounted on glass slides (mounting medium 0.25% gelatin in Tris buffer), dried overnight, and coverslipped. In most cases, visualization of the immunostaining was improved by processing the sections with nickel-enhanced DAB (0.05% DAB, 0.01% H_2O_2, 0.04% ammonium nickel sulphate in phosphate buffer).

Tract-tracing experiments

In vivo technique. All experiments were carried out under surgical anesthesia with MS222. The following tracers were used as retrograde and anterograde tracers: horseradish peroxidase (HRP, Boehringer), biotinylated dextran amine (BDA, 10kD-Molecular Probes D-1956), and rhodamine dextran amine (RDA, Molecular Probes D-1817). HRP was applied iontophoretically (a 15% HRP solution in phosphate buffer injected for 10 minutes using 5-8 μA positive pulsed current, 7 secs on/7 secs off), or as dry crystals onto a fine tungsten needle, to the DCN (five cases), to the most lateral part of the torus semicircularis (two cases), or to the thalamus (three cases). BDA was recrystallized from distilled water onto fine tungsten needles and applied to the proximal stumps of cut trigeminal nerves (four cases) or third spinal dorsal roots (three cases). Previous experiments (Nikundiwe et al. 1982, *Xenopus laevis*; M. Muñoz et al. 1991, *Rana perezi*) in which HRP was applied to thoracic and lumbar dorsal roots were used for the analysis of DCN projections of the more caudal dorsal roots. Additionally, material in which HRP or BDA was applied to the proximal stumps of the facial, glossopharyngeal, and vagal nerves, could be analyzed. Alternatively, BDA was injected iontophoretically as a 10% solution in phosphate buffer, into the DCN (three cases), cervical dorsal horn (three cases), the cerebellum (three cases), and thalamus (four cases). Survival times varied from 5 to 10 days. Subsequently, the animals were re-anesthetized and perfused through the heart with isotonic saline followed by a fixative containing 4% paraformaldehyde for the BDA experiments, 1.5% paraformaldehyde and 2% glutaraldehyde for the HRP cases. The brain and spinal cord were removed,

postfixed for 2-4 hours, and embedded in gelatin or polyacrylamide. Sections were cut transversally or horizontally at 40 µm on a freezing microtome. Histochemistry for HRP followed the heavy metal intensification of the diaminobenzidine (DAB)-based HRP reaction product (Adams, 1981). For visualizing BDA, an avidine biotine complex (Vectastain ABC Elite Kit, Vector Laboratories) was used. Some BDA-reacted sections were rinsed and further processed for calbindin D-28K or parvalbumin immunohistochemistry as described above. The black colour of the BDA labeling contrasts with the calbinding protein labeling stained brown by using the diaminobenzidine reaction *without* heavy metal intensification. RDA, recrystallized from distilled water onto sharp tungsten needles, was applied to the thalamus and to the torus semicircularis. After survival times of 2-4 days, animals were re-anesthetized with an overdose of MS222, and perfused with 0.1M phosphate buffer (pH 7.4) followed by a fixative containing 4% paraformaldehyde in phosphate buffer. The brain and (rostral) spinal cord were taken out, embedded in polyacrylamide, left overnight in 15% saccharose in 0.1M phosphate buffer, and cut transversally on a freezing microtome at 40 µm. They were mounted in glycerin-gelatin and viewed with a Zeiss fluorescence microscope with appropriate filter combinations.

In vitro technique. In 10 young adult *Xenopus laevis*, an *in vitro* approach was used, based on Cochran et al. (1987). The animals were deeply anesthetized with a 0.2% solution of MS222 and perfused with iced Ringer solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose; pH 7.4). The brains were removed, submerged in the same iced Ringer solution, and cut at middiencephalic or midmesencephalic levels. Applications of 3kD BDA (Molecular Probes, D-7135), recrystallized at the tip of sharp tungsten needles, were made at the ventral thalamus (5 cases) or the torus semicircularis (5 cases). The brains were kept for 5-18 hours at room temperature in continuously oxygenated Ringer solution (pH 7.4) with carbogen, and subsequently processed as described for the *in vivo* BDA experiments.

RESULTS

Delineation and (immuno)histochemical characterization of the anuran dorsal column nucleus

Cytoarchitecture. No distinct dorsal column nucleus (DCN) or nucleus funiculi dorsalis could be distinguished in most cytoarchitectonic studies of the anuran brain stem (e.g., Ariëns Kappers and Hammer, 1918; Zeehandelaar, 1921; Opdam et al., 1976). Therefore, since Woodburne's (1939) Marchi studies, the anuran DCN is defined as the site of termination of dorsal funicular fibers in the caudal brain stem, rather than as a cytoarchitectonic entity. Nevertheless, Nissl-stained sections of the brain stem at obex levels allow the delineation of a DCN (Fig. 1), although labeling of spinal primary afferent projections to the obex level by cobalt staining (Antal et al., 1980), HRP (Nikundiwe et al., 1982) or BDA (see Fig. 6) much more clearly delineates the DCN.

In *Xenopus laevis*, medial ('gracile') and lateral ('cuneate') compartments of the DCN can be distinguished above and lateral to the distinct solitary tract (Nikundiwe et al., 1982; Nikundiwe and Nieuwenhuys, 1983). A dorsal indentation suggests such a subdivision. Medially, the DCN is difficult to

distinguish from the nucleus of the solitary tract, and laterally it is very poorly segregated from the nucleus of the descending tract of the trigeminal nerve. Both compartments of the DCN consist of small (8-10 μm) and medium-sized (15 μm) multipolar elements. The medial, gracile part begins at the level of the second spinal nerve and extends into the brain stem, where it is situated dorsal and dorsolateral to the solitary tract (Fig. 1A,B). The lateral, cuneate part extends further rostrally than the gracile part, and borders on the nucleus of the descending trigeminal tract. In *Rana perezi*, like in *R. esculenta* (see Antal et al., 1980), the segregation of the DCN from the surrounding cell structures such as the nucleus of the descending trigeminal tract and the nucleus of the solitary tract is also rather poor. In *R. perezi*, the cell area dorsal and lateral to the solitary tract only occasionally shows an indentation allowing the distinction of medial and lateral compartments in the DCN (Fig. 1C-F). These gracile and cuneate subdivisions rostrally extend to the level of the glossopharyngeal nucleus where they are slightly more laterally located since in that position the most medial part of the rhombencephalic alar plate is occupied by the caudal pole of the vestibular nuclear complex and related descending vestibular root fibres (Fig. 1C,D). Lateral to the DCN cells, the cells in the dorsolateral position of the alar grey are mingled with afferent fibers of the Vth, VIIth, IXth and Xth cranial nerves forming the descending tract of the trigeminal nerve. These dorsolateral alar grey cells can be regarded as a component of the nucleus of the descending trigeminal tract.

In *Xenopus laevis* and to a lesser extent also in *Rana perezi*, the cell area above and lateral to the solitary tract at the obex level is composed of two DCN compartments, the lateral of which fades into the nucleus of the descending trigeminal tract.

Chemical neuroanatomy

NADPH-diaphorase histochemistry

In the caudal part of the rhombencephalic alar plate in *Rana perezi*, NADPHd-positive neurons were observed in the DCN, in the adjacent descending trigeminal nucleus, and in the nucleus of the solitary tract (Fig. 2A,B). In *Xenopus laevis*, the caudal lateral line nucleus was labeled as well. In the DCN, a cluster of NADPHd-positive neurons was found, the dendrites of which are directed dorsally toward the dorsal funiculus. It should be noted, however, that NADPHd-positive neuron populations are more distinct in the nucleus of the solitary tract, mainly in its medial and ventromedial parts below the solitary tract, and in the nucleus of the descending trigeminal tract (see Fig. 2A,C). By combining NADPHd staining with immunohistochemistry against tyrosine hydroxylase (TH), the NADPHd-positive part of the nucleus of the solitary tract was shown to be intermingled with the catecholaminergic cells (see González and Smeets, 1991) situated ventral to the solitary tract. In the caudal part of the rhombencephalic alar plate of both anuran species studied, NADPHd-positive fibers were found predominantly in two bundles, i.e., the descending trigeminal tract and the solitary tract. In the solitary tract, NADPHd-positive fibers were observed in its most dorsal part. Dorsal to the DCN the rostral continuation of labeled dorsal and dorsolateral (tract of Lissauer) funicular fibers can be observed. Heavy staining within these funiculi is present in the spinal cord.

Immunohistochemical data

The distribution of the calcium-binding proteins calbindin D-28k (Calb) and parvalbumin in the DCN is shown in figures 3 and 4. In *Rana perezi*, in the DCN area two Calb-positive neuron populations were distinguished (Fig. 3A-C): the first group coincides with the nucleus of the solitary tract; the second group is located in the dorsolateral grey, and is composed of round or oval-shaped, small and medium-sized cells with processes directed dorsolaterally into the dorsolateral funiculus. The highest density of this cell population was observed at the obex level and in the rostral spinal cord. This cell population forms part of the nucleus of the descending trigeminal tract, which was verified in experiments in which BDA was applied to the trigeminal nerve. A close relationship was observed between labeled trigeminal afferents and this Calb-positive cell group at the obex level. In young adult *Xenopus laevis*, a similar pattern of Calb-immunoreactivity was observed (Fig. 4A,D,E), although caudal to the obex the number of Calb-positive neurons in the nucleus of the descending trigeminal tract was much higher. In both anuran species, hardly any Calb-positive neurons were found in the DCN. In the dorsal horn of the spinal cord, however, an abundant Calb-positive cell population was observed.

In striking contrast to the lack of Calb-positive neurons in the DCN, in both anuran species a distinct parvalbumin (Parv)-positive DCN cell population was observed, particularly in *Xenopus laevis* (Fig. 4A-C). Apart from the DCN, Parv-positive neurons were observed in the reticular formation, octavolateral area, rostral part of the nucleus of the solitary tract, trigeminal nuclear complex, and dorsal and ventral horn of the spinal cord. In *Rana perezi*, relatively few Parv-positive DCN neurons were observed, spread throughout the nucleus. The dendrites of these rather large cells are mainly directed dorsally or laterally into the adjacent white matter. In some sections more dorsally located and smaller Parv-positive cells were observed as well. In young adult specimens of *X. laevis*, many more Parv-positive neurons were observed in the DCN. Even a clear segregation into a medial and a lateral component could be observed (see Fig. 4A-C). More ventrally located neurons probably form part of the nucleus of the solitary tract. In experiments in which the immunostaining against parvalbumin was combined with a BDA application to the third dorsal root, BDA-labeled endings close to the somata of Parv-positive neurons were observed, suggesting that they receive primary spinal afferents.

The data presented suggest that parvalbumin can be used as a marker for the DCN in anurans, and that calbindin D-28k almost certainly can not. However, since calbindin D-28k is abundantly present in the nucleus of the solitary tract as well as in the nucleus of the descending trigeminal tract, it can help to delineate the lateral and medial borders of the DCN.

In the caudal part of the medulla oblongata, scattered GABAergic neurons were observed in the alar plate. They are more densely grouped in two different locations: one in the nucleus of the solitary tract, the other in the DCN and the adjacent nucleus of the descending trigeminal tract. In the DCN, small, round or oval-shaped, neurons were found (see Fig. 5A,B). Figure 5C shows a control experiment. In the DCN of the anuran species studied no glycine-immunoreactive neurons were observed. In *Rana perezi*, however, some large glycinergic neurons were observed just ventrolateral of the DCN (see Fig. 5A,D). These bipolar neurons are oriented

dorsoventrally with processes directed into dorsal and ventral directions.

Tract-tracing experiments

In order to characterize the afferent and efferent connections of the DCN in *Rana perezi* and *Xenopus laevis*, horseradish peroxidase (HRP) histochemistry and biotinylated dextran amine (BDA) immunodetection were used. Additionally, the fluorescent tracer rhodamine amine (RDA) was used as a retrograde tracer. Even though the iontophoretic injections of the tracers were rather small, particularly the BDA injections, it was nearly impossible to restrict the application site to the DCN. Medially located injections often partially involved the nucleus of the solitary tract, whereas more lateral injections included part of the nucleus of the descending trigeminal tract. It is therefore not possible to fully discriminate the connectivity of the DCN using only this type of tracer application. Subsequent retrograde tracing experiments (HRP, BDA and RDA) were used to confirm the projections from the DCN, whereas anterograde tracing experiments were accomplished to corroborate and describe the terminal fields of the efferent connections of the DCN. Since the results in both species are largely comparable, in the following section the general pattern of the connections of the anuran DCN will be described. Only when differences are found between the species, this will be mentioned separately.

Afferent connections

The afferent projections to the DCN were studied by application of tracers to the cervical, thoracic or lumbar spinal dorsal roots, and to the roots of the trigeminal, facial, glossopharyngeal and vagal nerves. Additionally, after injections of tracers into the DCN, the cells of origin of non-primary afferent projections could be studied. Immunohistochemical data on afferent connections of the DCN will be discussed.

Ascending primary afferents. In all experiments of tracer application to a dorsal root, the labeled afferent fibers bifurcate into ascending and descending tracts upon entering the spinal cord. Two components are present, i.e., a medially located bundle of thick fibers that enters the dorsal funiculus, and a more laterally situated group of fibers within the dorsal portion of the dorsolateral funiculus. Both fiber systems project to widespread spinal and supraspinal regions. In the obex region the fibers in the dorsal funiculus are somatotopically organized. Fibers originating at lumbar segments occupy positions medial to those of thoracic origin. The most laterally located fibers in the dorsal funiculus enter at cervical levels. In the experiment shown in figure 6, BDA was applied to the proximal end of the cut third dorsal root. In such experiments the lateral compartment of the DCN could be clearly delineated (see Fig. 6A,D). Ascending collaterals from the large medial component of the cervical dorsal roots ascend in the lateral part of the dorsal funiculus. Those of Lissauer's tract reach the obex level via the dorsolateral funiculus. Lissauer's tract could be traced as far rostrally as the rostral pole of the nucleus of the solitary tract. By comparing the labeling pattern obtained after BDA application of the proximal end of the cut trigeminal nerve (Fig. 6A,B) to the pattern after labeling the third dorsal root, it seems likely that the sites of termination of cervical dorsal root fibers and trigeminal afferents are largely segregated. At DCN levels, however, thin trigeminal fibers not only innervate the nucleus of the descending trigeminal

tract, but also the DCN as delineated by dorsal root projections.

These ascending spinal projections clearly outline the DCN. In *Xenopus laevis*, the terminal fields marking the DCN were found from the level of the second spinal nerve up to the entrance of the vagal nerve. In *Rana perezi*, a similar rostral extent was observed but its caudal end coincides with the end of the hypoglossal nucleus. The terminal fields in this area largely resemble the organization of the fibers in the dorsal funiculus. Thus, medially situated axons from lumbar and thoracic dorsal root ganglion cells terminate on medial cells in the DCN, while laterally located fibers arising from cervical dorsal root ganglion cells end on more lateral cells, with a certain degree of overlap in the projection. This gracile- and cuneate-like organization of the DCN is similar in both anuran species.

Immunohistochemical studies showed that substance P- and CGRP-immunoreactive fibers are present in the tract of Lissauer. Some of these peptidergic fibers leave this tract and innervate the lateral part of the DCN (Fig. 7A). In addition to this peptidergic primary afferent projection to the DCN, the DCN is innervated by Leu-enkephalin, neuropeptide Y (NPY) and serotonin-immunoreactive fibers. Leu-enkephalin immunoreactive terminal structures were found in the dorsal grey at obex levels and in a thin rim around the solitary tract. NPY-immunoreactive fibers enter the DCN from the dorsal aspect of the lateral funiculus and form varicose fibers and terminal boutons. Serotonin-immunoreactive fibers rather strongly innervate the DCN and adjacent structures such as the nucleus of the solitary tract and the nucleus of the descending trigeminal tract (Fig. 7B).

Afferents from the Vth, VIIth, IXth and Xth cranial nerves. The application of anterogradely transported tracers to the proximal stumps of severed trigeminal, facial, glossopharyngeal and vagal nerve roots resulted in the labeling of their primary afferent projections. Apart from projections to their specific targets, all these nerves share a component of afferent fibers which, on entering the medulla, turn caudally in the lateral aspect of the rhombencephalon and continue into the spinal cord. These fibers course in the descending trigeminal tract and they constitute thin varicose fibers at DCN levels, which project not only to the laterally located nucleus of the descending trigeminal tract or to the commissural nucleus of the solitary tract but also to the DCN itself (Fig. 6B,C).

Non-primary DCN afferents. Following HRP or BDA injections into the DCN (see Figs. 8, 9), various non-primary DCN afferents were demonstrated. Non-primary ascending spinal projections arise throughout the spinal cord. Most of these cells were found ipsilaterally in the dorsal grey (Fig. 7C), but also some contralateral cells, as well as some cells in the intermediate and ventral grey were found (Fig. 7D). It should be noted that medially located injections resulted in the labeling of a higher population of lumbar and thoracic spinal cells, whereas more laterally placed injections labeled mainly cervical spinal cells. Other afferent projections to the DCN arise from the brain stem. After injections of retrograde tracers into the DCN, labeled cells were found, ipsilaterally, in the nucleus of the solitary tract, and, bilaterally, in the nucleus cerebelli. Bilaterally labeled cells were found in the reticular formation at levels between the VIIth and IXth motor nuclei, and in the ventral nucleus of the VIIIth nerve. A small population of labeled cells was present in the

raphe zone at levels between the entrance of the VIIIth and the IXth nerve roots. This projection from the raphe nucleus, presumably serotonergic, is in line with the rather strong serotonin-immunoreactive innervation of the DCN (see Fig. 7B).

Efferent projections: ascending

Anterograde tracing experiments

1) *The medial lemniscus.* In a first set of experiments, unilateral applications of the tracers HRP or BDA were made dorsally into the obex region (Figs. 8, 9). These applications clearly involved the DCN and the anterogradely labeled fiber projections were easily observed. In all cases, a distinct contralateral ascending system from the DCN was present, i.e., the medial lemniscus. Its axons could be traced from the injection site ventrally and medially, decussating to the contralateral side beneath the central canal (Figs. 8L, 9M), then turning rostrally into the ventral tegmentum. As the medial lemniscus ascends in the rhombencephalon, it smoothly swings to more dorsolateral positions. All through the medulla, the medial lemniscus gives off thin varicose fibers to various parts of the rhombencephalic reticular formation (Figs. 8J,K, 9I-L). A few smooth fibers run dorsally into the octavolateral area, and some enter the granular layer of the cerebellum (Figs. 8I, 9I). Rostrally, fine varicose fibers are observed ventrolateral to the isthmic nucleus. At caudal mesencephalic levels, the fibers turn dorsally along the lateral aspect of the midbrain and most of them bend medially, where they terminate in the torus semicircularis (Figs. 8F,G, 9F,G, 10C,D). The principal, magnocellular, and commissural nuclei receive only a sparse DCN projection, but the laminar nucleus is densely innervated, mainly in its lateral portion. A few fibers pass to the contralateral commissural and principal nuclei of the torus semicircularis. In *Rana perezi* medial lemniscal fibers do not reach the mesencephalic tectum, while in *Xenopus laevis* the intermediate and deep tectal layers are innervated. These rather thick medial lemniscal fibers innervating the tectum mesencephali often give off thin collaterals that terminate in the laminar nucleus of the torus semicircularis (Figs. 9E-G, 10C). In both species, at more rostral mesencephalic levels, the anterodorsal and anteroventral tegmental nuclei as well as the red nucleus and the interstitial nucleus of the fasciculus longitudinalis medialis are innervated by medial lemniscal fibers (Figs. 8E,F, 9E,F).

At rostral mesencephalic levels, scattered labeled fibers distribute to the pretoral grey, and, in *Xenopus laevis*, also to the pretectal grey (Figs. 8E, 9E). Beyond the midbrain, both the dorsal and ventral thalamic areas are innervated by medial lemniscal fibers (Figs. 8A-D, 9A-D). A few thin, varicose fibers innervate the ventral parts of the posterior and central dorsal thalamic nuclei, whereas the ventromedial thalamic nucleus and the posterior tubercle are far more densely innervated. The fibers reaching the ventromedial nucleus pass through the dorsal and ventral parts of the ventrolateral thalamic nucleus and varicosities are also found among its cells. Apart from a few fibers reaching the anterior nucleus of the dorsal thalamus (in two cases in *Rana perezi*), no labeling was found more rostrally in the anterior diencephalon or in the telencephalon in any of the cases.

2) *Extralemniscal ascending projections.* Apart from the medial lemniscus, the DCN gives rise to a distinct ipsilateral ascending projection (Figs. 8I-K, 9I-L). Due to their proximity, the ascending primary afferent spinal fibers bypassing the injection site were

most likely to be involved. Such fibers are known to project to the octavolateral area and the cerebellum (see Antal et al., 1980; Nikundiwe et al., 1982). Additionally, adjacent cell groups such as the nucleus of the descending trigeminal nucleus and the nucleus of the solitary tract might have incorporated the tracer from the injection sites. Therefore, ipsilateral projections from the DCN are difficult to demonstrate in anterograde experiments.

Retrograde tracing experiments

In order to verify whether these ascending projections really arise in the DCN, in both anuran species injections of HRP, BDA or RDA were placed into the thalamus, torus semicircularis and cerebellar region.

1) *Thalamic applications.* In this group of experiments, a retrograde tracer was applied to the thalamus in such a way that both the dorsal and ventral thalamus were implicated. Retrogradely labeled cells in the region of the DCN formed a mixed population of irregular, large cells, and round, small cells (Fig. 11A,D). Although the majority of the cells were located contralaterally, a minor component of ipsilateral cells was also present. The dendrites of these cells are long and directed both dorsally and ventrolaterally, reaching the dorsal and the dorsolateral funiculi, respectively. Their axons were followed into the contralateral medial lemniscus. In addition, a few cells were labeled bilaterally in the dorsolateral descending trigeminal tract. In *in vitro*-BDA experiments in young adult *Xenopus laevis* a similar pattern of labeling was observed (Fig. 12A,C).

2) *Toral applications.* When the injection sites were limited to the torus semicircularis, neurons were retrogradely labeled within the DCN. They were particularly found on the contralateral side, although an ipsilateral component was present as well. Two distinct cell groups were observed in *Rana perezi* (Fig. 11B,E). The first one is made up of large cells with a minor component of small cells located in the dorsalmost grey. They possess several processes extending into the dorsal fiber layer. Their axons course ventromedially, cross the midline and form part of the medial lemniscus. The second group of labeled cells is located in the lateral marginal zone of the dorsal grey from the level of the obex to the second spinal segment. These are large bipolar and irregular cells with long processes directed mainly to the dorsal part of the lateral funiculus and into the dorsal funiculus while their axons do participate in the medial lemniscus. In addition, retrogradely labeled cells were always found in the ipsilateral descending nucleus of the trigeminal nerve following toral injections. After similar toral injections in *Xenopus laevis*, retrogradely labeled cells in the DCN form a band positioned from dorsomedial to ventrolateral above the solitary tract. The most dorsally located cells possess dendrites extending into the dorsal funiculus, whereas more ventrolateral cells have dendrites reaching the dorsal aspect of the lateral funiculus. Some neurons were observed with dendrites reaching both the dorsal and dorsolateral funiculi (Fig. 12D). In *in vitro* experiments, BDA was applied to the torus semicircularis of *Xenopus laevis*. The pattern of labeling in the DCN is shown in Fig. 12B,D,E.

3) *Cerebellar applications.* In experiments with tracer applications into the lateral portion of the cerebellar plate, the underlying cerebellar nucleus and the adjacent grey were mostly implicated as well. At the obex region, these applications resulted in the

labeling of three cell groups. Most labeled cells were found at the ventromedial margin of the caudal extent of the fasciculus solitarius, on both sides of the medulla, probably due to the uptake of the tracer by fibers projecting from the nucleus of the solitary tract to the nucleus visceralis secundarius (parabrachial region). In the nucleus of the descending trigeminal tract labeled cells were found as well, mainly ipsilateral to the application site. The third group of retrogradely labeled cells was found, bilaterally, in the DCN (Fig. 11C,F). These cells were mainly found ipsilaterally. Their axons seem to run together with the ascending primary afferent fibers from the spinal dorsal roots.

Efferent projections: descending

In experiments with BDA or HRP applications into the DCN region anterogradely labeled axons could be followed from the injection site caudalwards into the spinal cord. These fibers course via the ipsilateral dorsal funiculus, and form fine arborizations of thin varicose fibers terminating among the cells in the dorsal horn throughout the cord, but particularly at cervical levels. A sparse bilateral innervation of the intermediate and ventral zones was also observed. These fibers could, however, represent fibers by-passing the injection site. Therefore, spinal injections with retrograde tracers were studied. A small population of cells, scattered in the area of the ipsilateral DCN, was always labeled after injection of the various spinal segments (Fig. 10G).

DISCUSSION

In the present study the organization, immunohistochemical characterization, and more particularly, the fiber connections of the anuran DCN were investigated. Although it is obvious that the anuran DCN remains a rather ill-defined area in the caudal part of the rhombencephalic alar plate, and no selective markers for the DCN other than its labeling by primary afferents from the spinal cord are available, NADPH-diaphorase staining and immunohistochemical staining of calcium-binding proteins and various neurotransmitters certainly help in delineating and characterizing the DCN. Since no clear cytoarchitectonic separation of the DCN into a medial, 'gracile' nucleus and a lateral, 'cuneate' nucleus is obvious, the term 'dorsal column nucleus' is preferred.

The NADPH-diaphorase (NADPHd) histochemical technique, known to stain specific neurons (Thomas and Pearse, 1964), can selectively stain particular populations of neurons in a Golgi-like manner (Scherer-Singler et al., 1983). Throughout the brain NADPHd and nitric oxide synthase (NOS) localizations are identical (Bredt and Snyder, 1992). Therefore, NADPHd can be used as a marker for NOS. Nitric oxide probably plays a major role as a neuronal messenger (Bredt and Snyder, 1992; Meller and Gebhart, 1993; Schuman and Madison, 1994). The presence of NADPHd-positive cells and fibers in the mammalian spinal cord (Valtschanoff et al., 1992) suggests that nitric oxide may be involved in spinal sensory processing. In the rat DCN, Valtschanoff et al. (1993) found that most NOS-positive neurons are also immunoreactive for GABA, but not for the excitatory transmitters glutamate and aspartate. Moreover, since NOS-positive neurons could not be labeled retrogradely from the thalamus or spinal cord, they are probably local circuit neurons (Valtschanoff et al., 1993). In the anuran species studied, NADPHd-positive neurons were found in the DCN, but

especially in the adjacent nucleus of the solitary tract and the descending nucleus of the trigeminal nerve in keeping with data in mammals (e.g., Leight et al., 1990; Vincent and Kimura, 1992; Dohrn et al., 1994; Takemura et al., 1994). Since no double labeling studies for GABA or excitatory transmitters were carried out, it remains to be analyzed whether these NADPHd-positive neurons are local circuit neurons or give rise to efferent projections such as the medial lemniscus.

Calcium-binding proteins such as calbindin D-28k, calretinin, and parvalbumin are found in certain subpopulations of neurons in the central and peripheral nervous system (Baimbridge et al., 1982; Garcia-Segura et al., 1984; Braun, 1990; Celio, 1990; Ren and Ruda, 1994). They even label entire pathways, sometimes whole functional systems (Celio, 1990; Andressen et al., 1993). In mammals, calcium-binding proteins like calbindin and parvalbumin show a preferential distribution in somatosensory structures, including the DCN (e.g., Celio, 1990; Rausell and Jones, 1991a,b; Rausell et al., 1992; Menetrey et al., 1992a,b; Maslany et al., 1992; Ren and Ruda, 1994). Parvalbumin (Parv) appears to be abundant in the pathway for epicritic sensibility, i.e., the dorsal column-medial lemniscal system, calbindin D-28k (Calb) occurs in the whole taste pathway of rats (Celio, 1990). In rats, Calb-positive neurons are found in certain laminae of the dorsal horn (see Antal et al., 1990; Ren and Ruda, 1994) including the cells of origin of ascending spinal projections (Menétrey et al., 1992b), in the sensory trigeminal nuclei as well as in the gracile and cuneate nuclei (Celio, 1990). In rats, Menétrey et al. (1992a) showed that Calb-positive neurons form a major part of the solitary and trigeminal projection systems. In the trigeminal system of monkeys, both proteins are differentially expressed in the ascending trigeminothalamic projections to the ventral posteromedial (VPM) nucleus (Rausell and Jones, 1991a,b). Antisera to parvalbumin and calbindin mark VPM rods and matrix which receive principal and spinal trigeminal input, respectively. A similar segregation has been demonstrated for the ascending somatosensory projections from the spinal cord (Rausell et al., 1992): A non-nociceptive Parv-positive dorsal column-medial lemniscal projection terminates in cytochrome oxidase (CO)-rich domains of the ventral posterolateral thalamic nucleus (VPL) where Parv-positive neurons are found. Nociceptive Calb-positive spinothalamic fibers terminate in CO-poor domains of the VPL where Calb-positive cells are present (Rausell et al., 1992).

Against this background, the presence of calbindin D-28k and parvalbumin in the anuran DCN was studied. It appeared that in the alar plate of the caudal rhombencephalon, Calb-positive neurons were found particularly in the nucleus of the solitary tract and in the descending nucleus of the trigeminal nerve continuing into the dorsal horn of the spinal cord. In both anuran species studied, hardly any Calb-positive neurons were found in the DCN itself. This pattern of distribution of Calb-positive neurons suggests that in anuran amphibians, as in mammals, Calb could be restricted to the nociceptive part of the somatosensory system including neurons in the dorsal horn of the spinal cord, and the descending nucleus of the trigeminal nerve. In striking contrast, in both anuran species a distinct parvalbumin (Parv)-positive DCN population was observed, particularly in *Xenopus laevis*. Parv-positive neurons were found throughout the DCN, their dendrites were mainly directed dorsally or laterally into the adjacent dorsal funiculus. Immunostaining of parvalbumin can therefore be used

to delineate the anuran DCN. It should be noted, however, that the pattern of distribution of calcium-binding proteins in the rat DCN is quite different. Maslany et al. (1992) found both Calb- and Parv-positive neurons in the cuneate and gracile nuclei, although Parv-positive DCN cells were more numerous. The distribution of Parv-cells appeared to be similar to the known distribution of thalamic projection neurons.

In mammals, the presence of small GABAergic interneurons within the DCN has been extensively described (for reviews see Mugnaini and Oertel, 1985; Rustioni and Weinberg, 1989). Also glycinergic inhibitory effects within the DCN were observed. Does the anuran DCN contain GABAergic interneurons? In the present study small, round or oval-shaped GABA-immunoreactive neurons were observed. The pattern of labeling in other parts of the brain stem is comparable to that described by Franzoni and Morino (1989, *Rana esculenta*), who, unfortunately, did not include the most caudal part of the brain stem in their analysis. Double labeling studies, i.e. combinations with tract-tracing or NADPH-diaphorase, are needed to demonstrate whether these GABA-immunoreactive neurons in the anuran DCN are actually interneurons. In this respect, it should be noted that Pritz and Stritzel (1989a) suggested that the reptilian *(Caiman crocodilus)* DCN lacks glutamic acid decarboxylase (GAD)-immunoreactive neurons, indicating that the reptilian DCN – like the dorsal thalamus (see Pritz and Stritzel, 1988) – lacks local circuit neurons. A few glycinergic neurons were found at the ventrolateral border of the DCN. In the lamprey, such glycinergic neurons are known to inhibit reticulospinal neurons (Dubuc et al., 1993a,b). In mammals, glycinergic cells of different sizes have been observed in the gracile and cuneate nuclei (Porucho et al., 1992).

Even though cytoarchitectonic studies do not clearly define the anuran DCN, this nucleus is characterized by its somatotopic organization of primary afferent projections from the spinal cord (Antal et al., 1980, *Rana esculenta*; Nikundiwe et al., 1982, *Xenopus laevis*). Data in *Rana perezi* (M. Muñoz et al., 1991) indicate a similar pattern of arrangement, whereby primary afferents from lumbar and thoracic dorsal root ganglia innervate the medial, 'gracile' compartment of the DCN, while those from cervical ganglia innervate its lateral, 'cuneate' compartment as well as the spinal or descending trigeminal nucleus. The dorsal funicular projection continues rostrally to innervate the vestibular nuclear complex and, rather abundantly, the granular layer of the cerebellum (Antal et al., 1980; Székely et al., 1980). Fibers terminating in the vestibular nuclei and in the cerebellum arise from limb-innervating spinal ganglia (Antal et al., 1980; González et al., 1984). The non-primary spinal afferents or postsynaptic dorsal column system (PDCS) also appears to be arranged somatotopically. The presence of such a PDCS has now been demonstrated throughout terrestrial vertebrates (e.g., Rustioni, 1973; Angaut-Petit, 1975a,b; Rustioni and Kaufman, 1977; Bennett et al., 1984; Giesler et al., 1984; Funke, 1988; ten Donkelaar and de Boer-van Huizen, 1991; Pritz and Stritzel, 1994). In mammals, the cells of origin of these non-primary afferent projections to the DCN, or postsynaptic dorsal column neurons, have been shown to transmit nociceptive information (Uddenberg, 1968; Angaut-Petit, 1975b; Bennett et al., 1984; Kamogawa and Bennett, 1986), at least in cats.

The lateral part of the anuran DCN is innervated by fibers from the descending tract of the trigeminal nerve, arising from the descending part of the trigeminal, facial, glossopharyngeal and vagal nerves (Fig. 12; see also Rubinson and Friedman, 1977; Matesz and Székely, 1978; Fuller, 1979; Lowe and Russell, 1982; Altman and Dawes, 1983; Stuesse et al., 1984; Oka et al., 1987; González et al., 1993; M. Muñoz et al., 1994). In contrast, lateral line nerve projections, present in permanently aquatic species such as *Xenopus laevis*, strictly avoid the DCN (see Lowe and Russell, 1982; Altman and Dawes, 1983; Fritzsch et al., 1984; Will et al., 1985a).

The most lateral part of the anuran DCN is also innervated by substance P- and CGRP-immunoreactive fibers passing via the tract of Lissauer (see also Rosenthal and Cruce, 1985; Adli et al., 1988; Petkó and Sánta, 1992). In addition to this peptidergic primary afferent projection to the DCN, the anuran DCN is innervated by Leu-enkephalin, neuropeptide Y and serotonin-immunoreactive fibers in line with data by Ueda et al. (1984), Merchenthaler et al. (1989), and Lázár et al. (1990). This serotonergic and peptidergic innervation of the DCN is in line with immunohistochemical data in mammals (e.g., Steinbusch, 1981; Westman et al., 1984; Halliday et al., 1988; Ibuki et al., 1989; Tamatani et al., 1989; Conti et al., 1990; Fabri and Conti, 1990; Blomqvist and Broman, 1993). Since after tracer applications to the DCN retrogradely labeled neurons were observed in the (serotonergic – see Ueda et al., 1984) raphe nucleus, it seems likely that this nucleus is the source of the serotonergic innervation of the DCN. In rats, Willcockson et al. (1987) observed serotonergic terminals in apposition to neurons of the DCN that project to the thalamus, whereas in cats and monkeys, Blomqvist and Broman (1993) observed serotonergic input to DCN neurons projecting to various brainstem areas including pretectum, superior colliculus and pontine nuclei, related to motor processing.

Descending control of the DCN, so prominent in mammals (see Willis and Coggeshall, 1991 for review), seems to be rather restricted in anurans. After injections of tracers into the DCN, labeled cells were found bilaterally in the cerebellar nucleus, in the ventral nucleus of the VIIIth nerve and in the reticular formation at levels between the VIIth and IXth motor nuclei including the inferior raphe nucleus. In mammals, the transmission of sensory information through the dorsal column-medical lemniscus pathway is controlled by pathways from the cerebral cortex (e.g., Kuypers, 1958; Kuypers and Tuerck, 1964), the red nucleus (Edwards, 1972; Weinberg and Rustioni, 1989), vestibular nuclei (Weinberg and Rustioni, 1989), the cerebellum (Sotgiu and Cesa-Bianchi, 1972), and the reticular formation (Willcockson et al., 1987; Weinberg and Rustioni, 1989). Therefore, with the possible exception of the red nucleus, a comparable brain stem 'control' of the DCN is found in anurans.

A major part of the present study focused on the efferent connections of the DCN, more in particular on the targets of the medial lemniscus. The existence of a dorsal column-medial lemniscal system in amphibians remained a much debated question until the early 1980's. Subsequently, Vesselkin and co-workers (Vesselkin et al., 1971; Vesselkin and Kova´cevi´c, 1973), Silvey et al. (1974) as well as Neary and Wilczynski (1977), described a contralateral projection of the DCN or 'perisolitary band' (Neary and Wilczynski, 1977) to thalamic nuclei. With electrophysiological techniques, Urbán and Székely

(1982) noted slow negative potentials from the posterocentral nucleus of the thalamus in response to stimulation of the 2nd dorsal root, the dorsal column and the dorsal column nucleus. In the present study the course and site of termination of the medial lemniscus was shown by anterograde tracing (Figs. 8, 9), and its cells of origin by retrograde labeling of the DCN from its main targets, i.e., the ventral thalamus, the lateral part of the torus semicircularis and the cerebellar cortex (Figs. 11, 12). The use of a new and powerful anterograde tracer like BDA made it possible to identify even fine terminal fields and the scattered fibers in the thalamus. The data obtained are summarized in Fig. 13. Since it was hardly possible to restrict tracer applications to the DCN, in such injections the adjacent nucleus of the solitary tract and the descending nucleus of the trigeminal nerve as well as fibers of passage (e.g., spinal primary afferents to the cerebellum) might be involved. By retrograde labeling the origin of the medial lemniscal projections was verified. It should be emphasized that the ventrolateral part of the DCN found to project to the thalamus and, more in particular, to the torus, extends caudally as far as the second spinal segment. The dendrites of these cells are mainly directed to the dorsolateral funiculus, whereas their axons join the contralateral medial lemniscus. A certain similarity to the mammalian lateral cervical nucleus, known to receive somatosensory information via the spinocervical tract and projecting contralaterally via the medial lemniscus (see Willis and Coggeshall, 1991), seems likely.

The medial lemniscus could be traced throughout the brain stem and into the diencephalon. Along its course, the medial lemniscus gives off collaterals to various parts of the reticular formation, to the octavolateral area, and to the granular layer of the cerebellum. At mesencephalic levels, the medial lemniscus primarily innervates the lateral part of the torus semicircularis (also noted by Comer and Grobstein, 1981; Wilczynski, 1981; Neary and Wilczynski, 1986; Neary, 1988), and the anterodorsal and anteroventral tegmental nuclei as well as the red nucleus and the interstitial nucleus of the fasciculus longitudinalis medialis. While in *Rana perezi* medial lemniscal fibers do not reach the tectum mesencephali, in *Xenopus laevis* intermediate and deep tectal layers are innervated in agreement with retrograde tracer data (Wilczynski and Northcutt, 1977; Zittlau et al., 1988; Hofmann et al., 1990; Masino and Grobstein, 1990). Beyond the midbrain, both dorsal and ventral thalamic areas are innervated by the medial lemniscus. The ventral parts of the posterior and central nuclei of the dorsal thalamus are reached by a few thin, varicose, fibers, but the ventromedial thalamic nucleus and the nucleus of the posterior tubercle are far more densely innervated. In two cases in *R. perezi*, a few fibers also reached the anterior nucleus of the dorsal thalamus. No projections beyond the diencephalon were observed. Extralemniscal projections were found to the ipsilateral cerebellar cortex, confirming retrograde tracer data (González et al., 1984), and bilaterally to the spinal cord. The ipsilateral spinal projection from the DCN was previously observed in *Xenopus laevis* (ten Donkelaar et al., 1981).

Hence, the present study not only further substantiated the presence of a rather well-developed dorsal column-medial lemniscus system in anurans, but also showed that its mesencephalic and diencephalic targets are much more extensive and diverse than suggested in previous studies (Vesselkin et al., 1971; Silvey et al., 1974; Neary and Wilczynski, 1977; Comer and Grobstein, 1981;

Wilczynski, 1981; Forehand and Farel, 1982; Urbán and Székely, 1982; Neary, 1988). The anuran 'lemniscal pathway' appears to be basically similar to that of amniotes (reptiles: Ebbesson, 1978; Siemen and Künzle, 1994a; birds: Wild, 1989; mammals: e.g., Hazlett et al., 1972; Hand and van Winkle, 1977; Feldman and Kruger, 1980; Berkley et al., 1986; see also Willis and Coggeshall, 1991 for a summary of mammalian studies), although in mammals the widespread thalamic projections should be particularly emphasized. In the red-eared turtle, *Pseudemys scripta elegans*, Siemen and Künzle (1994a,b) noted a direct ascending projection from the most medial part of the DCN area, by-passing the thalamus, to the basal part of the telencephalon.

At first sight, the mesencephalic target of the anuran medial lemniscus seems to be quite different from the amniote mesencephalic target. For the opossum, RoBards et al. (1976) introduced the term 'intercollicular terminal zone' for the common target of projections from the dorsal column nuclei, spinal cord and sensorimotor cortex in the central midbrain. In reptiles, Ebbesson (1967, 1969) introduced the term 'intercollicular nucleus' for the mesencephalic target of ascending spinal projections. In this nucleus, a projection from the dorsal column nucleus terminates as well (Ebbesson, 1978; see also Belekhova et al., 1985; Pritz and Stritzel, 1989b). It seems likely that this intercollicular zone, nucleus or 'midbrain somatosensory area' (Pritz and Stritzel, 1989b), characterized by at least an input from the spinal cord and DCN, is a major integrative center of the somatosensory system. Pritz and Stritzel (1990) showed that the medialis complex in the dorsal thalamus is the thalamic target of the midbrain somatosensory intercollicular area. In anurans, the main midbrain target of the medial lemniscus is formed by the lateral part of the torus semicircularis (Comer and Grobstein, 1981; Wilczynski, 1981; Neary and Wilczynski, 1986; Neary, 1988; the present study). The anuran torus semicircularis is a major integrating center for a number of sensory and non-sensory afferents in addition to its auditory input, and may well serve a role similar to the one the tectum mesencephali serves for the visual system (Wilczynski and Capranica, 1984). It includes a laminar nucleus, a principal nucleus, a magnocellular nucleus, and two smaller nuclei (Potter, 1965), each of which receives a particular set of afferents (e.g., Wilczynski, 1988; Feng and Lin, 1991). Physiological studies (Comer and Grobstein, 1981) in *Rana pipiens* suggest a certain overlap of tactile and auditory information: the very dorsolateral torus is almost exclusively concerned with tactile information; auditory activity is most often found to be localized in central parts of the torus, but in between the two, multimodal (tactile and auditory) activity is found. Toral afferents also arrive from the vestibular (Wilczynski, 1981), and, in *Xenopus laevis*, from the lateral line system (Will et al., 1985b; Lowe, 1986; Zittlau et al., 1986). The laminar toral nucleus not only receives DCN efferents but also spinal (Ebbesson, 1976; A. Muñoz et al., in preparation) and trigeminal afferents (Comer and Grobstein, 1981; M. Muñoz et al., 1994), and so – at least partly – represents a midbrain somatosensory area. The multimodal laminar nucleus as well as the mainly auditory magnocellular nucleus extensively innervate the central and posterior nuclei (Frontera's nucleus posterocentralis; see Frontera, 1952) of the dorsal thalamus, whereas the ascending projections of the principal nucleus are restricted to the caudal part of the posterior thalamic nucleus (Hall and Feng, 1987; Feng and Lin, 1991). The laminar nucleus also innervates the ventromedial thalamic nucleus (Feng

and Lin, 1991; A. Muñoz and ten Donkelaar, unpublished observations), i.e., the main diencephalic target of the medial lemniscus (present study) as well as of the spinothalamic tract (A. Muñoz et al., 1994). The central thalamic nucleus extensively projects to the ipsilateral striatum (Vesselkin et al., 1980; Wilczynski and Northcutt, 1983a; Neary, 1988). Hence, this DCN – torus – central thalamic nucleus – striatal pathway is one way by which somatosensory information may reach the telencephalon.

Although the anuran dorsal column-medial lemniscus system is basically similar to that of amniotes, large differences are found with regard to the telencephalic targets of this pathway. Therefore, a few remarks on the telencephalic structures receiving somatosensory information in anurans seem appropriate. Physiological studies revealed somatosensory activity within the medial pallium (e.g., Supin and Gusel'nikov, 1964; Karamian et al, 1966; Northcutt, 1970; Vesselkin and Kova´cevi´c 1973), possibly relayed in the dorsal thalamus. Since the anterior thalamic nucleus is the only thalamic nucleus innervating the medial pallium (Scalia and Colman, 1975; Vesselkin and Ermakova, 1978; Kicliter, 1979; Neary, 1984; Northcutt and Ronan, 1992), somatosensory information to this pronounced telencephalic structure, also known as the archipallium (Ariëns Kappers et al., 1936; Clairambault and Derer, 1968) or the primordium hippocampi (Herrick, 1910; Hoffman, 1963), must relay in the anterior nucleus. However, since spinal afferents to the anterior thalamic nucleus – either via the spinothalamic tract (A. Muñoz et al., 1994) or via the dorsal column-medial lemniscal pathway (Neary and Wilczynski, 1977; present study) – are rather limited, alternative routes must be available, possibly via the posterior thalamic nucleus known to project to the anterior thalamic nucleus (Neary and Wilczynski, 1979; see also Neary, 1990; Northcutt and Ronan, 1992). It should also be noted that the dendrites of cells in the anterior thalamic nucleus to penetrate the central nucleus (Neary, 1990). Therefore, somatosensory information could reach the medial pallium via multisynaptic routes.

Another telencephalic structure in which somatosensory activity was recorded is the striatum (see Vesselkin et al., 1971; Vesselkin and Kova´cevi´c 1973). The anuran striatum receives a major thalamotelencephalic input from nuclei relaying sensory information from the midbrain roof, from the torus semicircularis, and from ventral diencephalic structures, receiving spinal and DCN-medial lemniscal afferents (Scalia and Colman, 1975; Vesselkin et al., 1980; Wilczynski and Northcutt, 1983a; Lázár and Kozicz, 1990). The striatum in amphibians appears to be able to influence the midbrain roof via the pretectum and various midbrain and isthmal nuclei (Wilczynski and Northcutt, 1983b). The anuran striatum plays a crucial role in processing sensory information as well as in coordinating all telencephalic output to lower brain stem motor centers. Both visual (Gruberg and Ambros, 1974) and auditory (Mudry and Capranica, 1980) activity was recorded from the striatum. Further, electrical stimulation of the sciatic nerve evoked potentials in the striatum (Vesselkin et al., 1971; Vesselkin and Kova´cevi´c, 1973). The sensory input to the striatum is relayed in the anterior division of the lateral thalamic nucleus (visual information: Lázár, 1969; Scalia, 1976), in the central thalamic nucleus (auditory and also somatosensory information: Hall and Feng, 1987; Feng and Lin, 1991; present study), and in the ventromedial thalamic nucleus (somatosensory information: Neary and Wilczynski,

1977; present study). In *Rana perezi*, tracer applications to the striatum showed a direct striatal projection arising from cells in the lateral aspect of the ventromedial thalamic nucleus (A. Muñoz, unpublished observations) in line with observations by Vesselkin et al. (1980) as well as by Lázár and Kozicz (1990). Vesselkin et al. (1980) also noted a direct striatal projection from the torus semicircularis, whereas Lázár and Kozicz (1990) found a few faintly labeled small cells in the nucleus of the posterior tubercle projecting to the lateral wall of the telencephalon including the striatum (see also Wilczynski and Northcutt, 1983a). Somatosensory information to the striatum may thus be relayed via the dorsal thalamus (the central thalamic nucleus), the ventral thalamus (the ventromedial thalamic nucleus), and via the nucleus of the posterior tubercle, a separate diencephalic region (see Neary and Northcutt, 1983). Which thalamic nuclei really relay somatosensory information to the striatum (and medial pallium) is now being studied in a series of double-labeling experiments.

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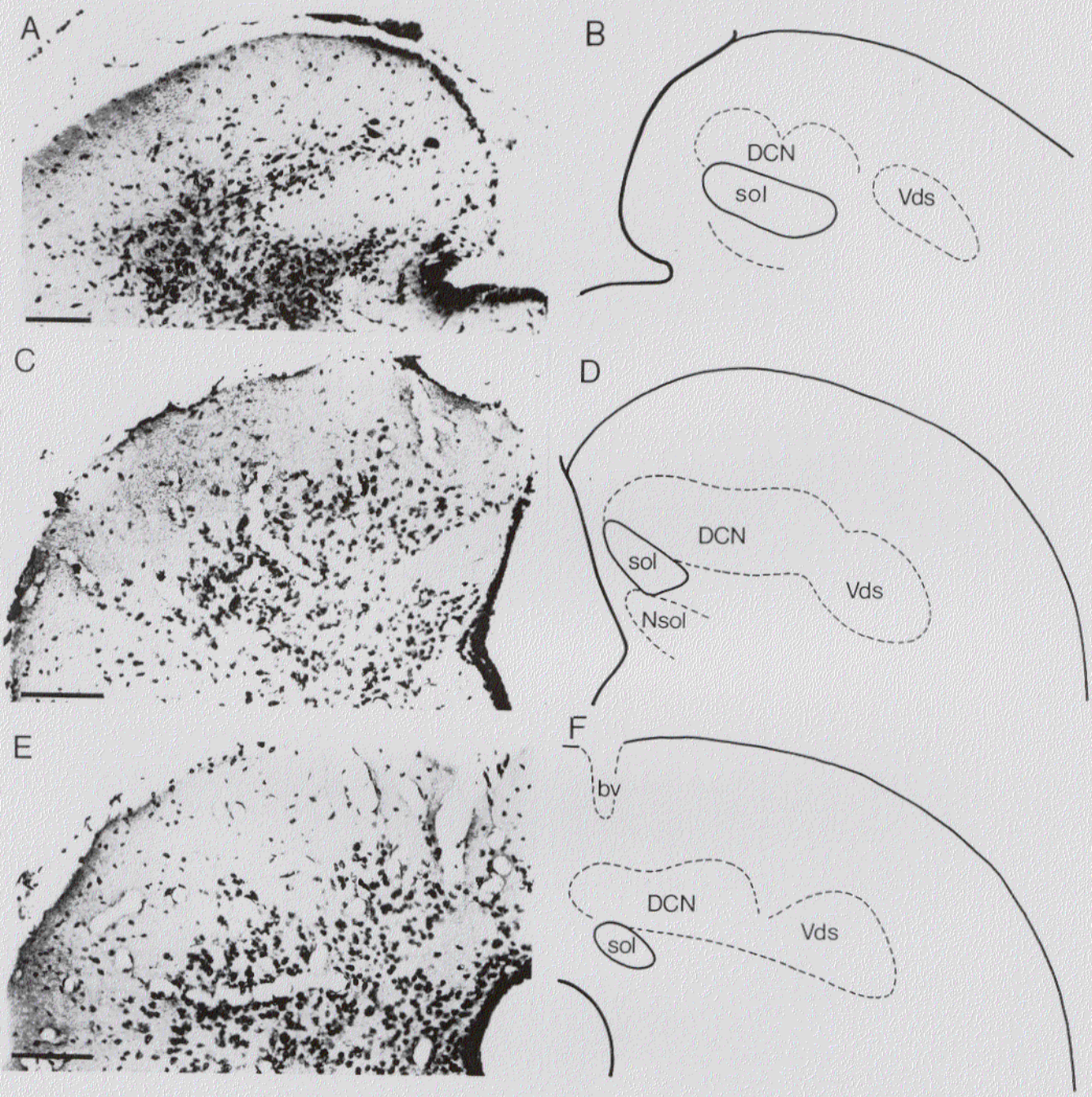

Fig. 1: Photomicrographs of Nissl-stained transverse sections and schematic drawings of the caudal part of the alar plate in *Xenopus laevis* (A, B) and in *Rana perezi* (C, F). Scale bars indicate 100 µm.

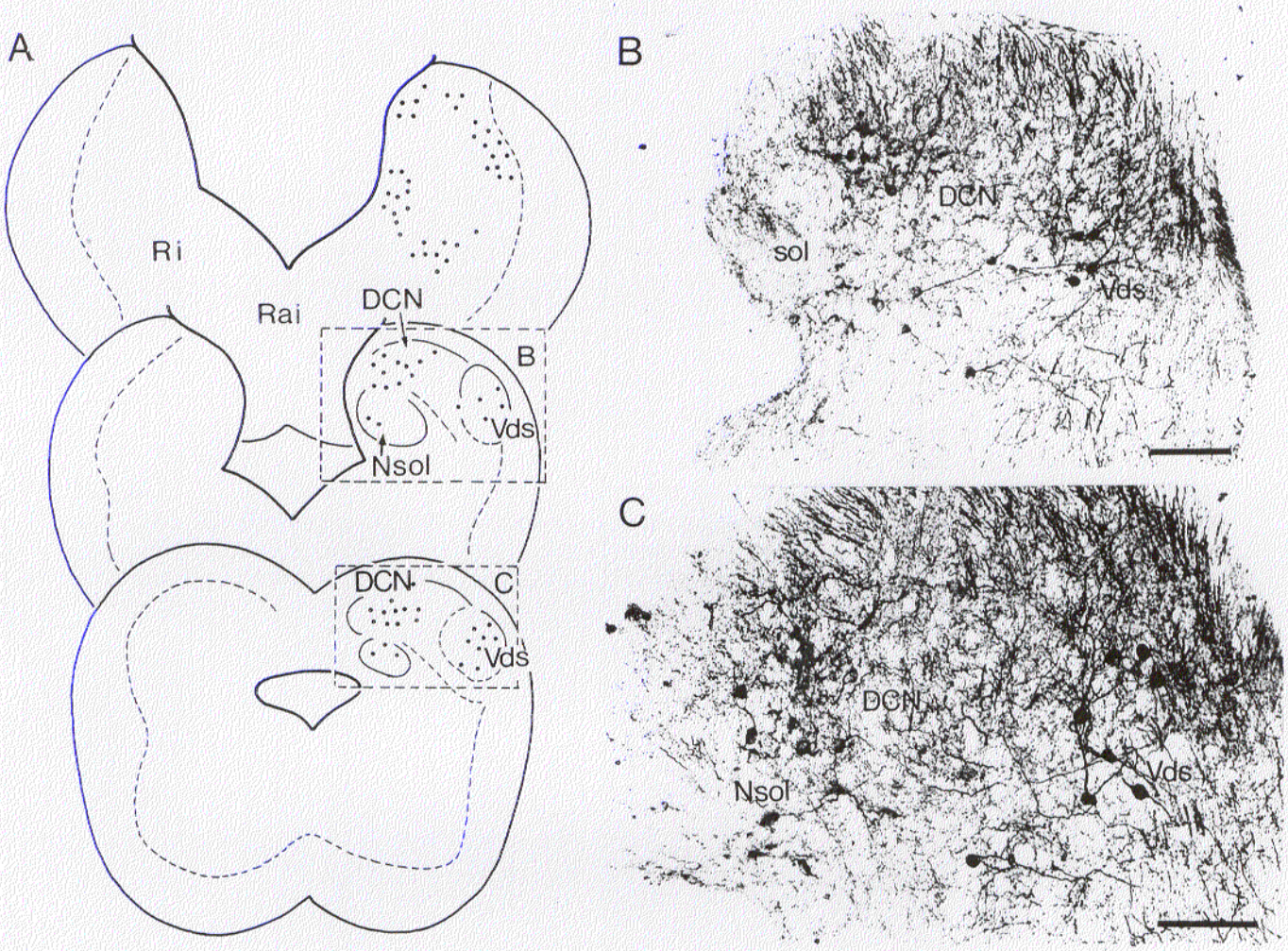

Fig. 2: A, The distribution of NADPH diaphorase-positive cells in the caudal part of the rhombencephalic alar plate of *Rana perezi*. B, C, Photomicrographs illustrating the medial (nucleus of the solitary tract), lateral (trigeminal) and dorsomedial (DCN) components. Scale bars indicate 100 µm.

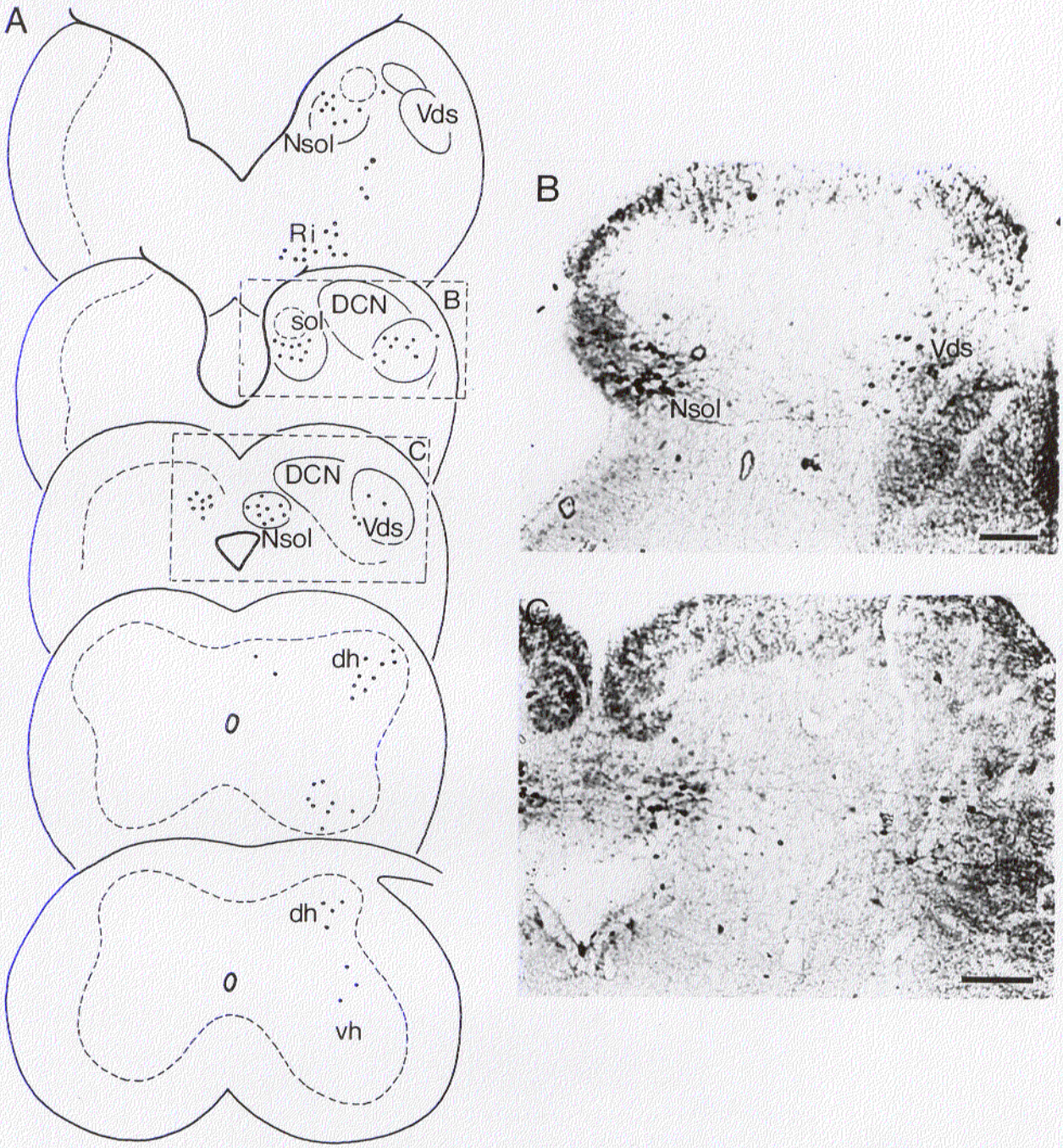

Fig. 3: A, B, C, The distribution of calbindin D-28k-positive neurons in the caudal part of the rhombencephalic alar plate and the rostral spinal cord of *Rana perezi*. Scale bars indicate 100 µm.

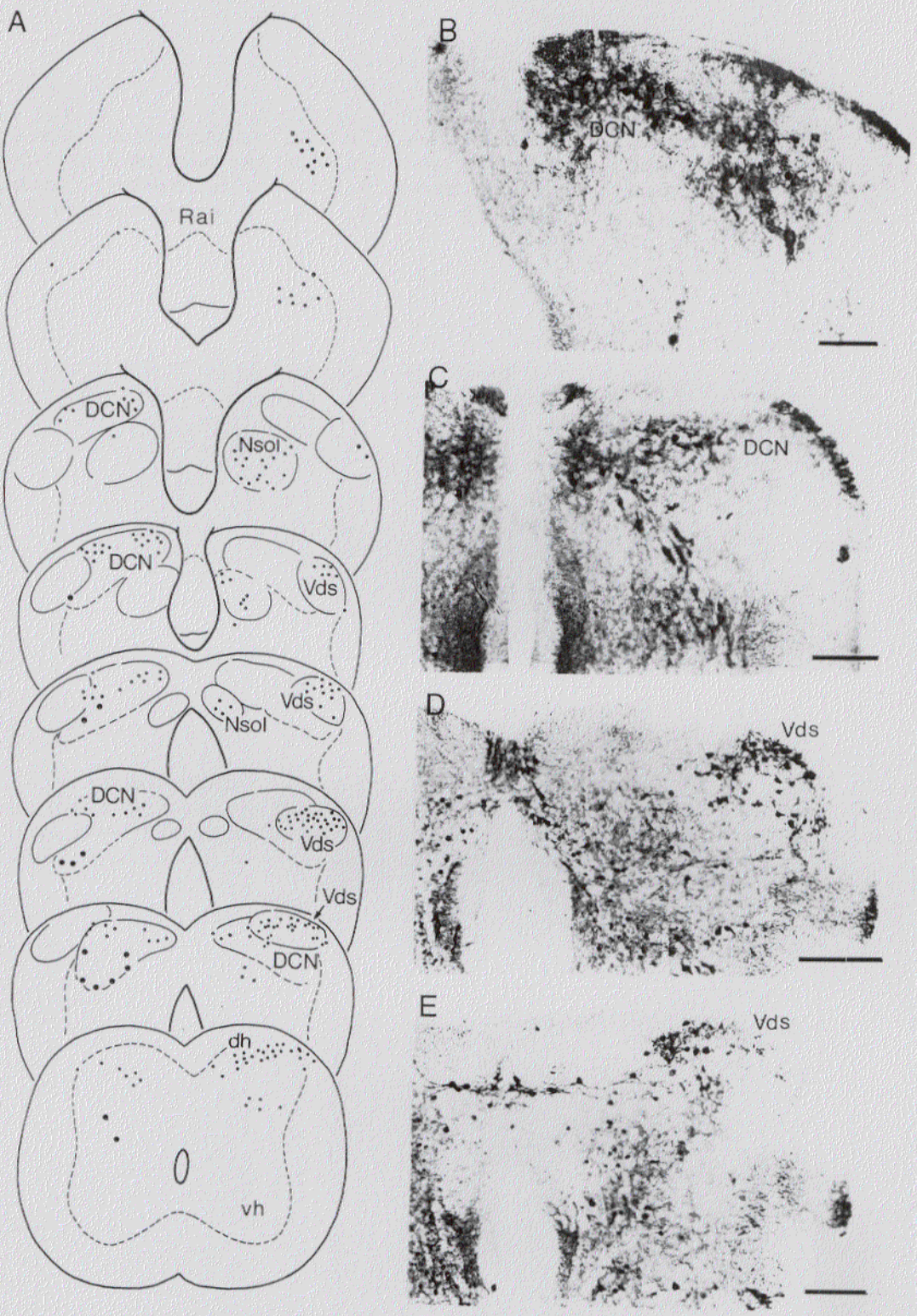

Fig. 4: A, The distribution of calcium-binding proteins in the caudal part of the rhombencephalon and most rostral part of the spinal cord of *Xenopus laevis:* on the left the distribution of parvalbumin-immunoreactive neurons, on the right the distribution of calbindin D-28k immunoreactive neurons. B-E, Photomicrographs of examples of parvalbumin (B, C) and calbindin (D, E) labeling. Scale bars indicate 100 µm.

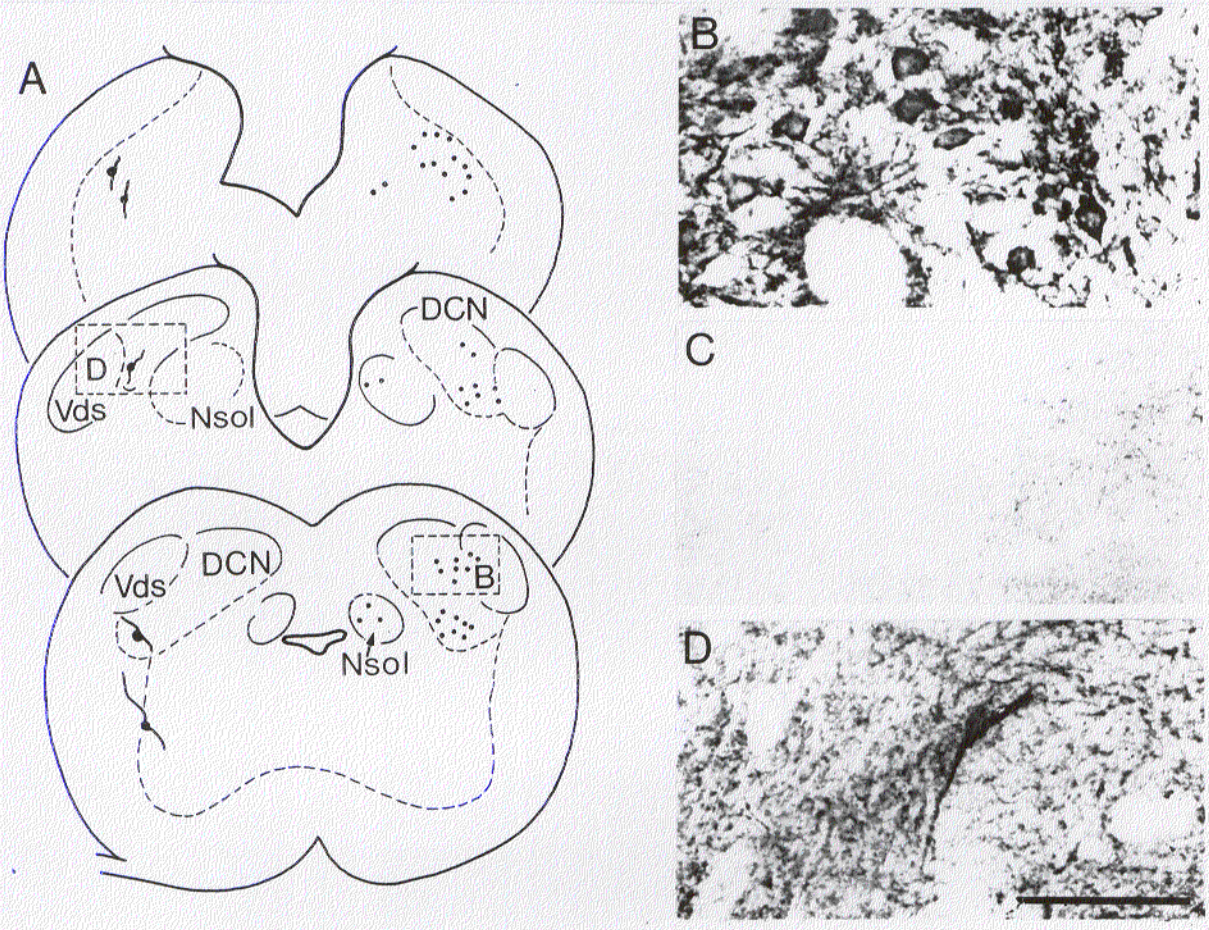

Fig. 5: A, The distribution of GABA-immunoreactive (on the right) and glycine-immunoreactive (on the left) neurons. In B, some GABA-immunoreactive neurons are shown, in C, the DCN area in a control experiment in which sections were stained with a solution of preimmune rabbit serum (1:1,000) instead of the rabbit anti-GABA antiserum, in D a glycinergic neuron. Scale bar for the photomicrographs indicates 100 µm.

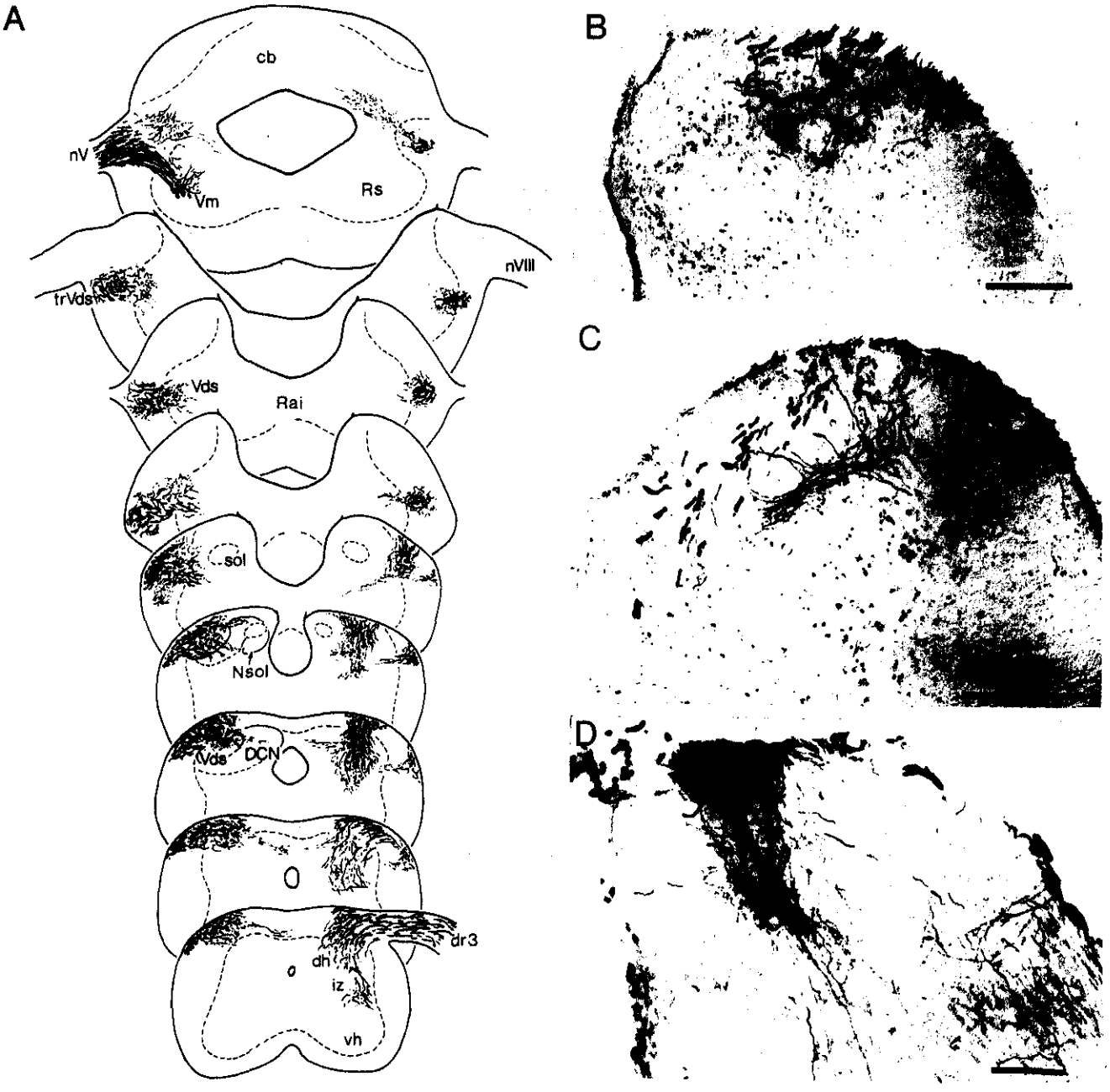

Fig. 6: A, Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Rana perezi* showing the distribution of biotinylated dextran amine (BDA)-labeled trigeminal (left side) and third dorsal root (right side) afferent fibers. B, C, Photomicrographs showing ipsilateral trigeminal (B) and glossopharyngeal (C) afferents to the DCN of *R. perezi*. D, Photomicrograph showing the termination pattern of BDA labeled brachial afferents of the third dorsal root at the rostral DCN of *R. perezi*. Scale bars indicate 100 μm.

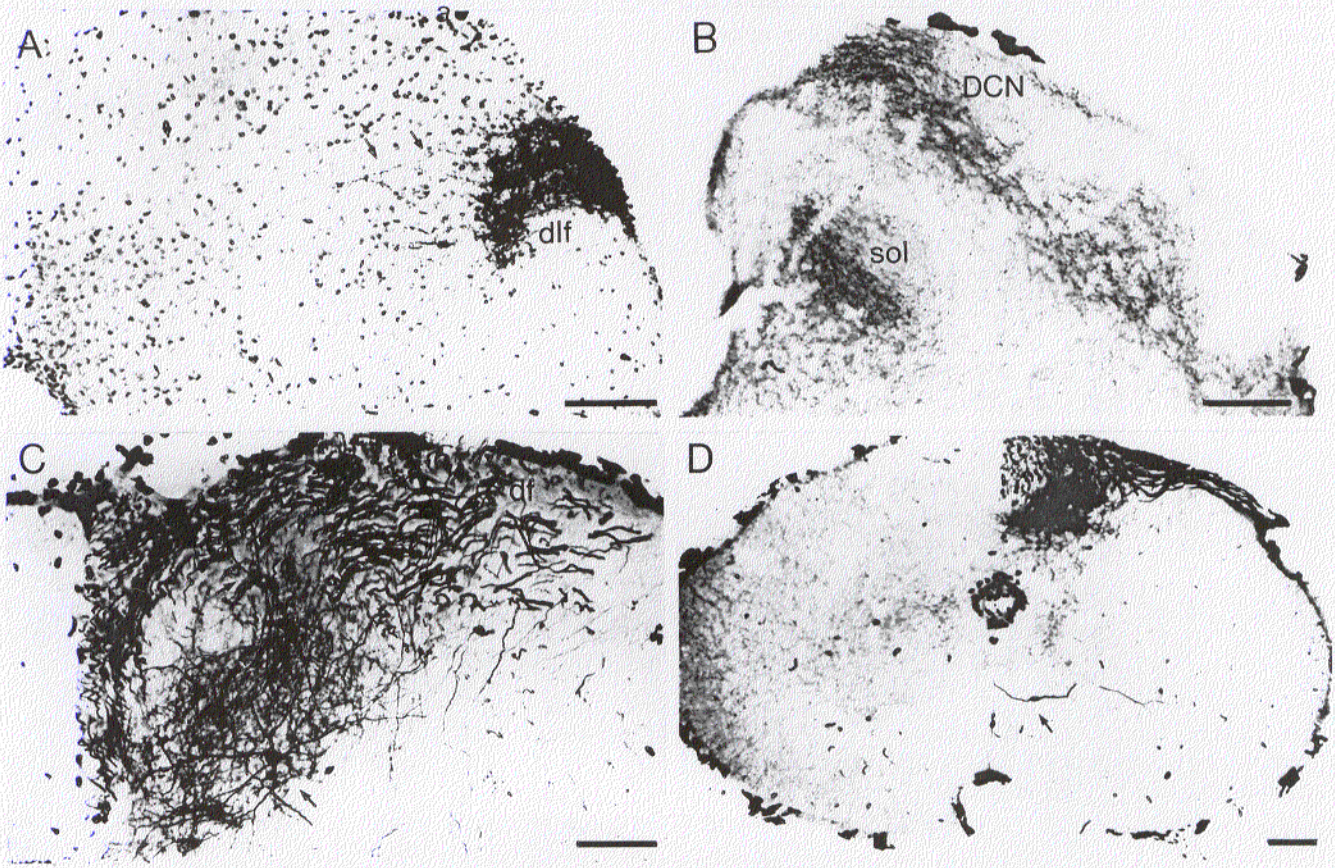

Fig. 7: A, B, Photomicrographs showing the substance P- (A, arrows) and serotonin-immunoreactive (B) innervation of the DCN in *Xenopus laevis* and *Rana perezi*, respectively. C, D, Photomicrographs showing (arrows) neurons of the postsynaptic dorsal column system in the cervical dorsal horn (C), and in the thoracic ventral horn (D) of *R. perezi*. Scale bars indicate 100 µm.

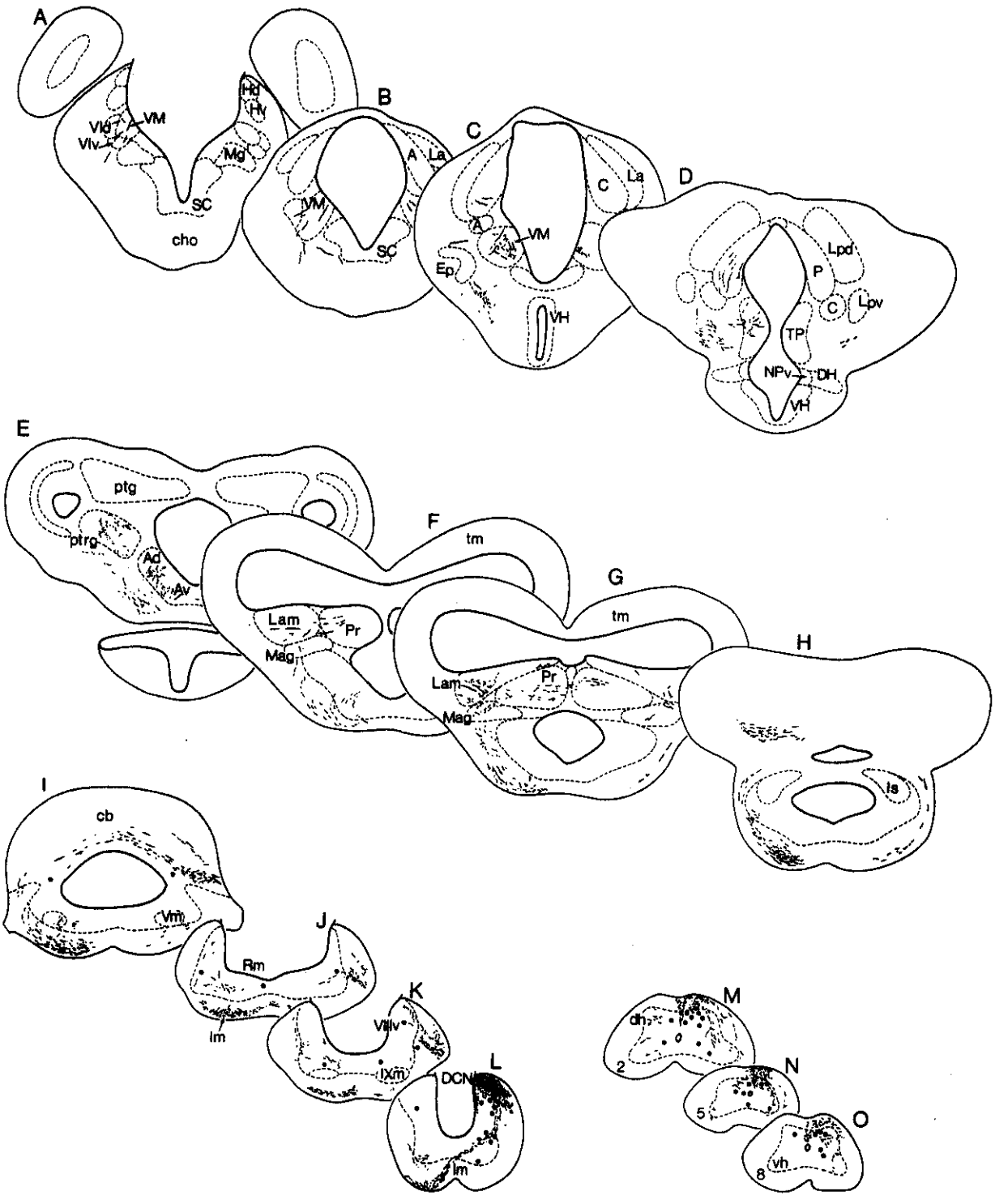

Fig. 8: Labeling observed after a BDA injection into the DCN of *Rana perezi* (for injection site see also Fig. 10A). In a series of transverse sections through the diencephalon (A-D), brain stem (E-L) and spinal cord (M-O) the pattern of anterogradely labeled fibers and retrogradely labeled cells (black dots) is shown.

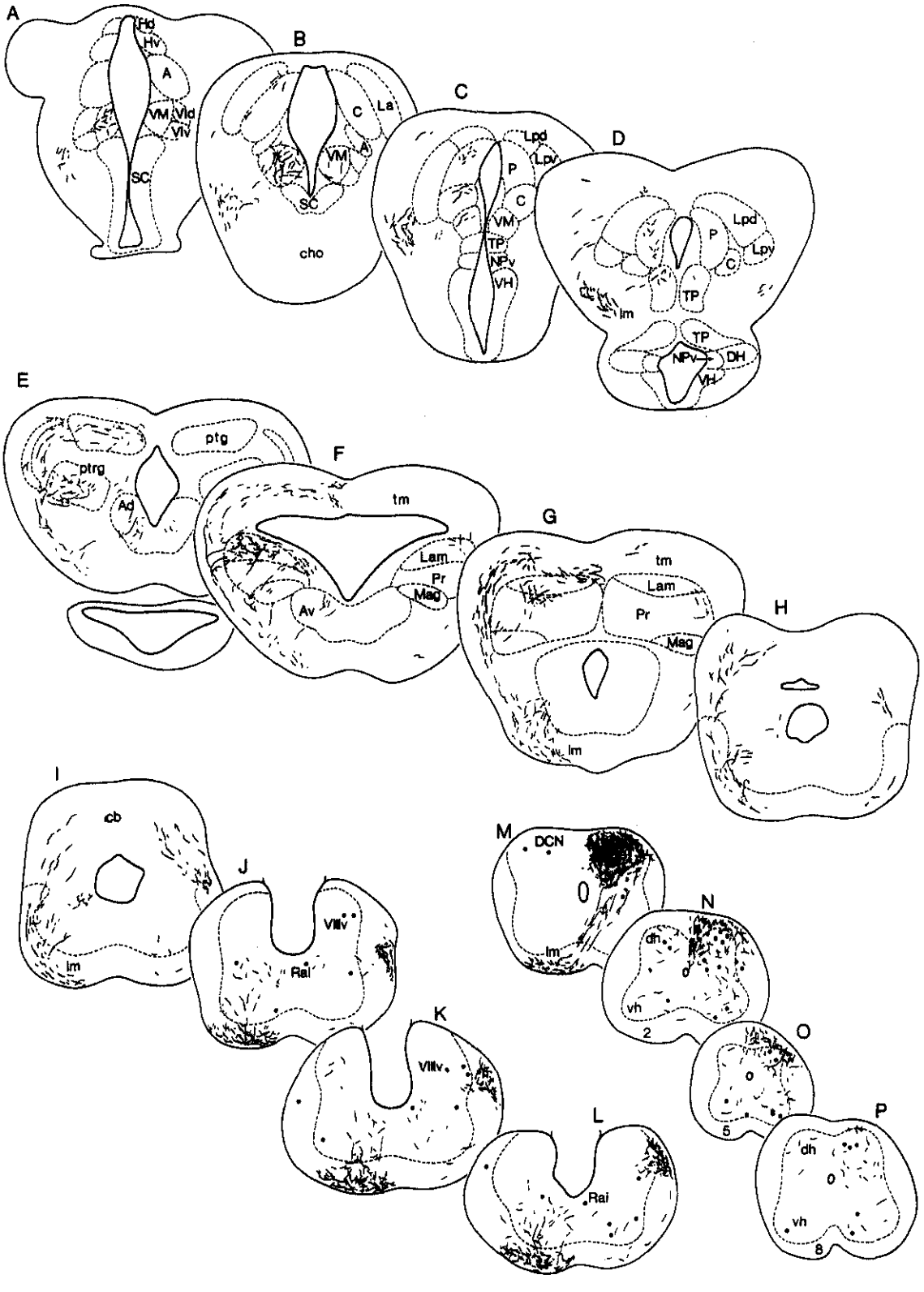

Fig. 9: Labeling observed after a BDA injection into the DCN of *Xenopus laevis*. In a series of transverse sections through the diencephalon (A-D), brain stem (E-M) and spinal cord (N-P) the pattern of anterogradely labeled fibers and retrogradely labeled cells (black dots) is shown.

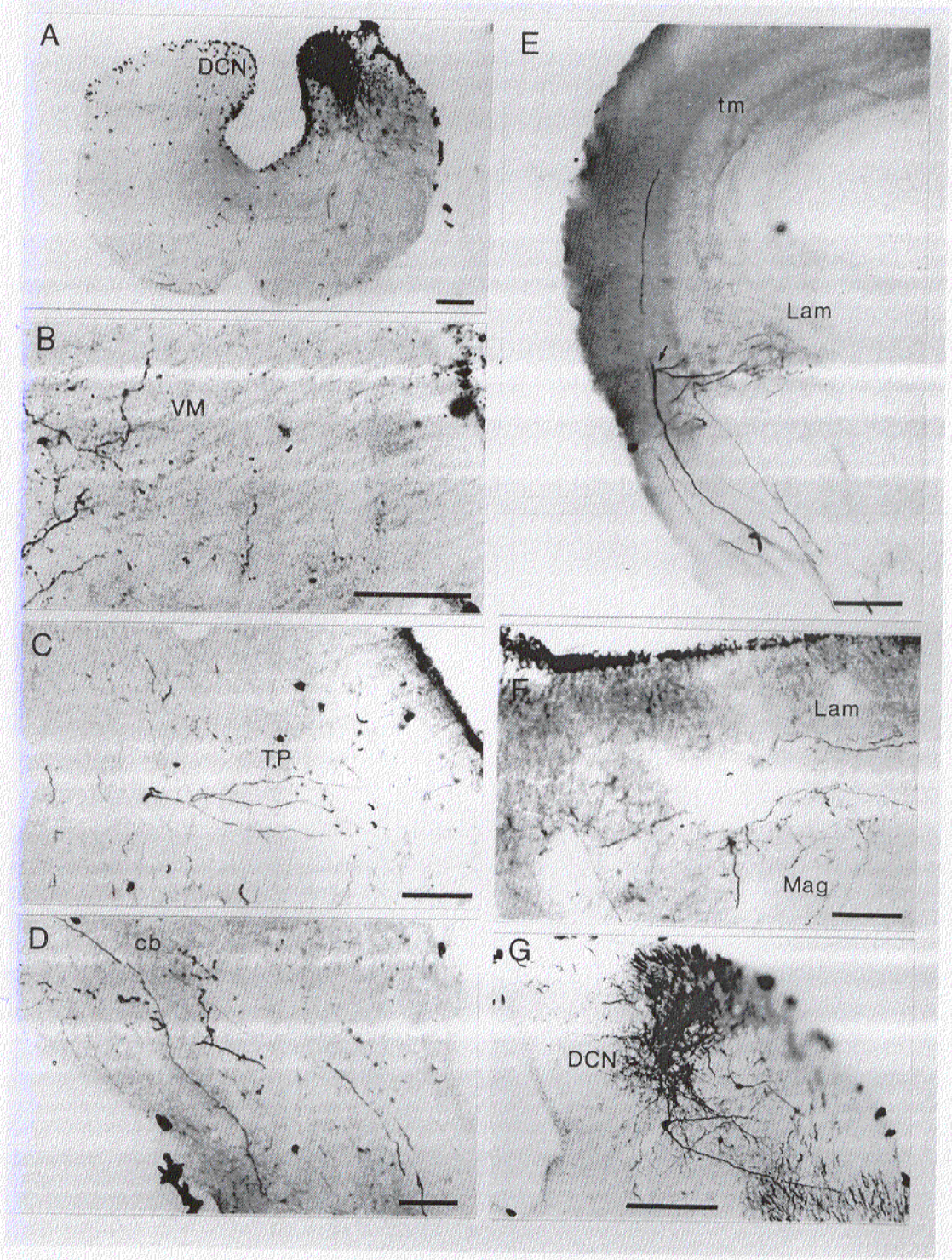

Fig. 10: Photomicrographs illustrating the labeling observed after BDA injections into the DCN (A-D, F) or spinal cord (G) of *Rana perezi*. In E anterogradely HRP labeled fibers from the DCN to torus and tectum in *Xenopus laevis* are shown; arrow points to a tectal axon giving off collaterals to the laminar nucleus of the torus. A: BDA injection site of the experiment shown in Fig. 8. B: Anterograde labeling in the contralateral ventromedial thalamic nucleus; C: Ibid., in the contralateral nucleus of the posterior tuberculum; D: Ibid., in the ipsilateral granular layer of the cerebellum; F: Ibid., in the laminar and principal nuclei of the contralateral torus semicircularis. In G: Retrogradely labeled cells in the DCN following a BDA application into the fourth spinal segment. Scale bars indicate 100 µm.

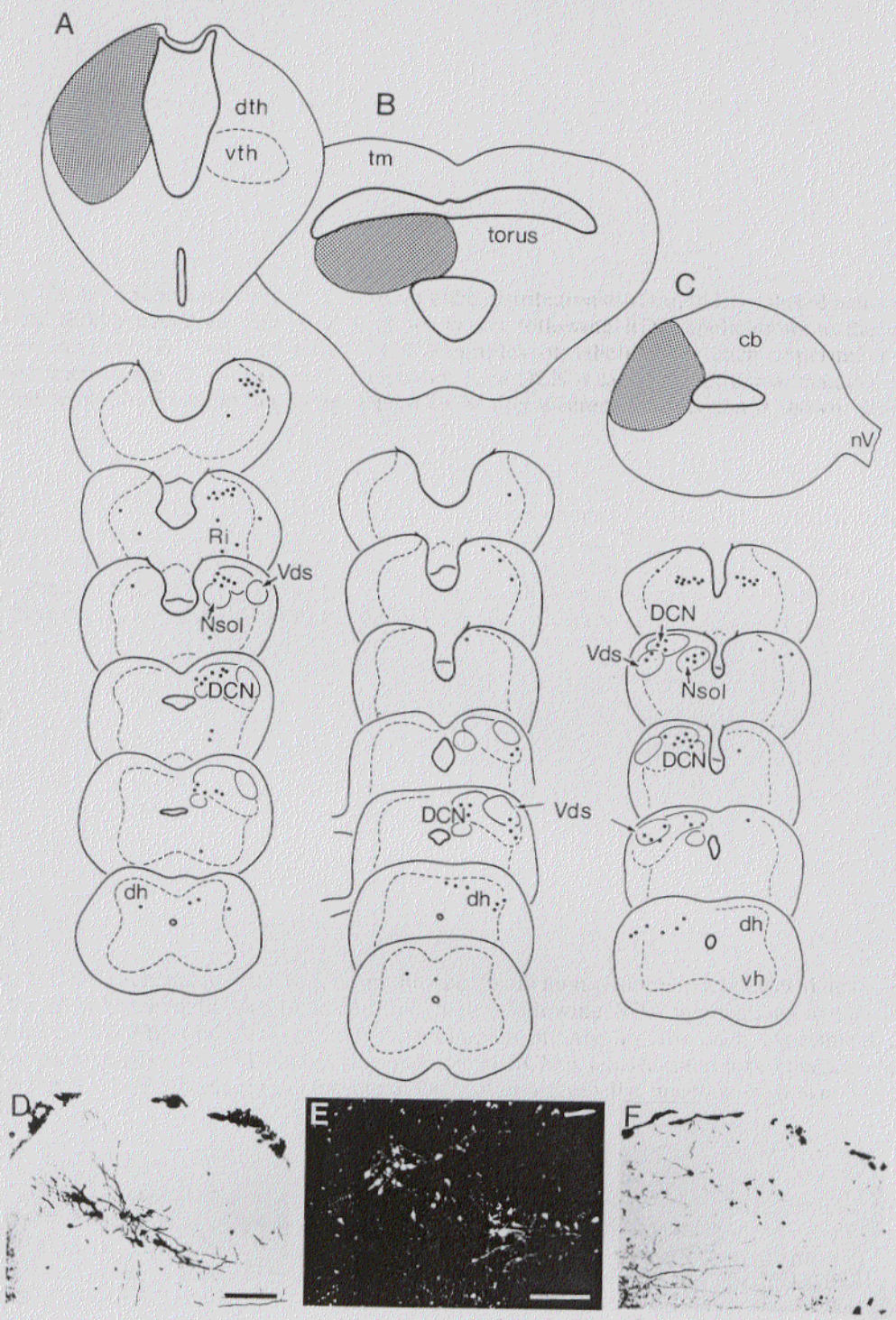

Fig. 11: A-C, Schematic drawings illustrating the distribution of retrogradely labeled neurons in the caudal part of the rhombencephalic alar plate of *Rana perezi* following BDA applications to the thalamus (A), torus semicircularis (B) and cerebellum (C). Examples of labeling for each experiment are shown in the photomicrographs D-F. In D and E the contralateral DCN is labeled after dorsal thalamic and toral injections, respectively. In F labeling in the ipsilateral DCN after a cerebellar injection is shown. Scale bars indicate 100 µm.

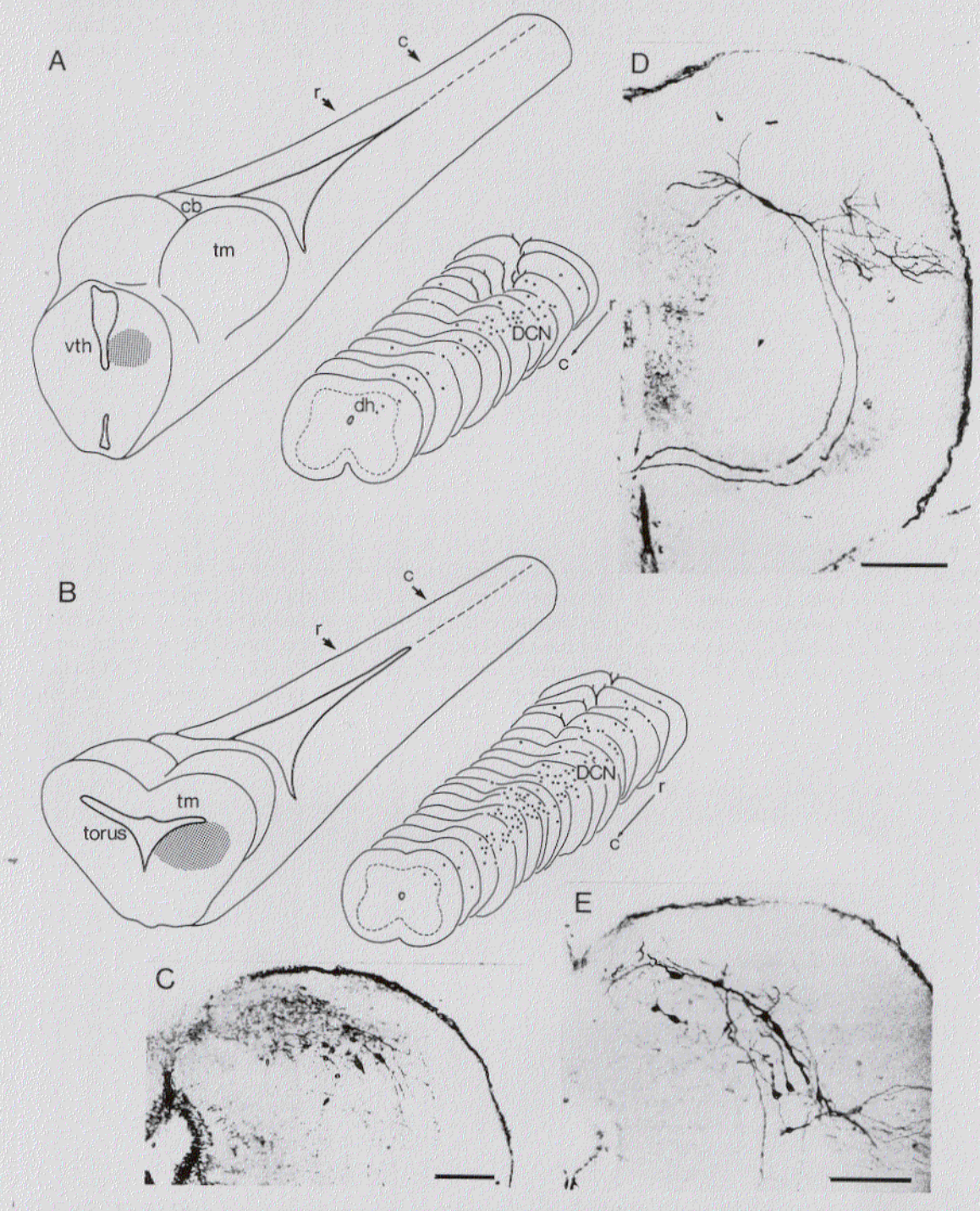

Fig. 12: A, B, Two *in vitro* experiments in *Xenopus laevis*. BDA was applied to the ventral thalamus (A) and torus semicircularis (B), respectively. In the photomicrographs C-E examples of labeling are shown: C, DCN neurons projecting to the contralateral ventral thalamus; D, E, DCN neurons projecting to the contralateral torus semicircularis. In D, one contralaterally projecting toral projection neuron at the ventrolateral aspect of the caudal DCN area with dorsally and ventrally oriented dendrites is shown; the arrow marks two axons crossing the midline to join the medial lemniscus. Scale bars indicate 100 μm.

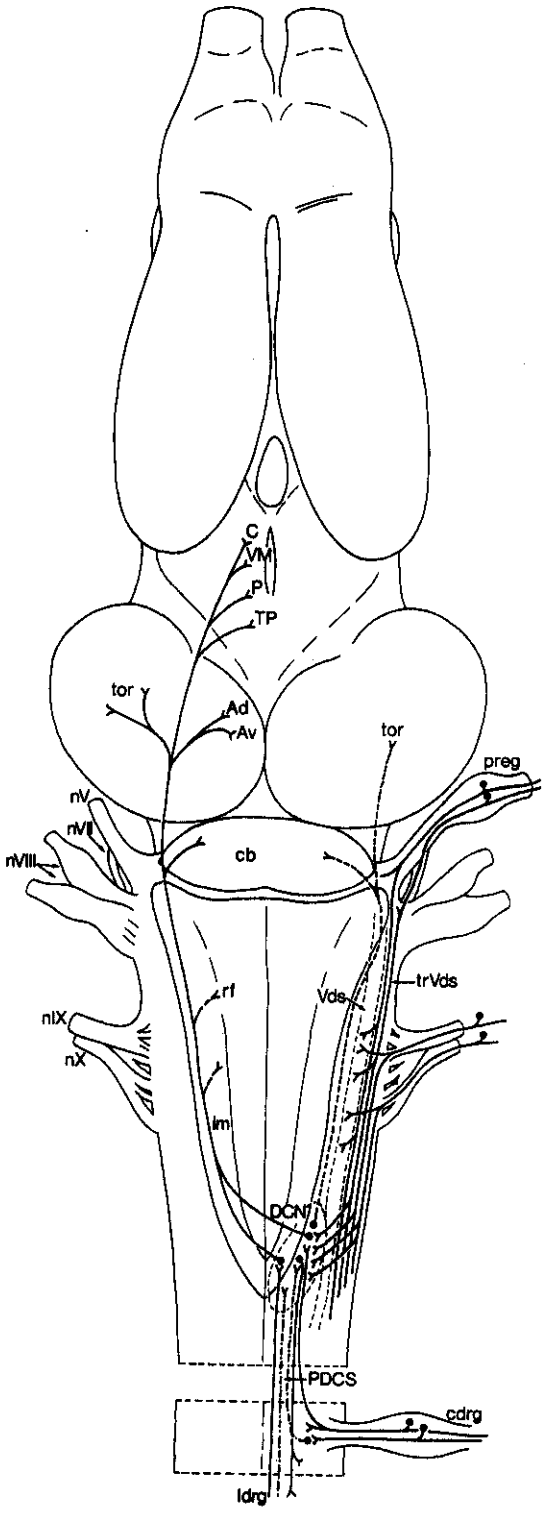

Fig. 13: Diagram summarizing the fiber connections of the anuran dorsal column nucleus shown in a dorsal view of the brain of *Rana perezi*.

LIST OF ABBREVIATIONS

A	anterior thalamic nucleus
Ad	anterodorsal tegmental nucleus
Av	anteroventral tegmental nucleus
bv	blood vessel
C	central thalamic nucleus
c	caudal
cb	cerebellum
cdrg	cervical dorsal root ganglion
cho	chiasma opticum
DCN	dorsal column nucleus
df	dorsal funiculus
DH	dorsal hypothalamic nucleus
dh	dorsal horn
dlf	dorsolateral funiculus
dr3	third dorsal root
dth	dorsal thalamus
Ep	posterior entopeduncular nucleus
Hd	dorsal habenular nucleus
Hv	ventral habenular nucleus
Is	nucleus isthmi
IXm	motor nucleus of the glossopharyngeal nerve
iz	intermediate zone
La	lateral thalamic nucleus: anterior division
Lam	laminar nucleus of the torus semicircularis
ldrg	lumbar dorsal root ganglion
lm	lemniscus medialis
Lpd	lateral thalamic nucleus: posterodorsal division
Lpv	lateral thalamic nucleus: posteroventral division
Mag	magnocellular nucleus of the torus semicircularis
Mg	magnocellular preoptic nucleus
Nsol	nucleus of the solitary tract
NPv	nucleus of the periventricular organ
nV	trigeminal nerve
nVII	facial nerve
nVIII	vestibulocochlear nerve
nIX	glossopharyngeal nerve
nX	vagal nerve
P	posterior thalamic nucleus
PDCS	postsynaptic dorsal column system
Pr	principal nucleus of the torus semicircularis
preg	preotic ganglion
ptg	pretectal grey
ptrg	pretoral grey
r	rostral
Rai	nucleus raphes inferior
Ri	nucleus reticularis inferior
Rm	nucleus reticularis medius
Rs	nucleus reticularis superior
SC	suprachiasmatic nucleus
sol	solitary tract
tm	tectum mesencephali
tor	torus semicircularis
TP	nucleus of the posterior tubercle
trVds	descending tract of the trigeminal nerve
VH	ventral hypothalamic nucleus
vh	ventral horn
Vds	nucleus of the descending tract of the trigeminal nerve
VIIId	descending nucleus of the VIIIth nerve
VIIIv	ventral nucleus of the VIIIth nerve
Vld	ventrolateral thalamic nucleus: dorsal part
Vlv	ventrolateral thalamic nucleus: ventral part
VM	ventromedial thalamic nucleus
Vm	motor trigeminal nucleus

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Evidence for an anuran homologue of the mammalian spinocervicothalamic system: An in vitro tract-tracing study in Xenopus laevis

5.3

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ABSTRACT

Evidence is presented for an anuran homologue of the mammalian spinocervicothalamic system. *In vitro* tract-tracing experiments with biotinylated dextran amine in *Xenopus laevis* show that ascending spinal fibers from all levels of the spinal cord, passing via the dorsolateral funiculus, terminate in a cell area ventrolateral to the dorsal column nucleus. This cell area can be considered a possible homologue of the mammalian lateral cervical nucleus. After tracer applications to the ventral thalamus or to the torus semicircularis (both targets for somatosensory projections), the anuran lateral cervical nucleus was retrogradely labeled contralateral to the application sites. Tracer applications to the dorsolateral funiculus at the obex level and rostral spinal cord resulted in labeling of the cells of origin of the anuran spinocervical tract. These were found, mainly ipsilaterally, in the ventral part of the dorsal horn, and

were rather evenly distributed throughout the spinal cord. These data suggest the presence of an anuran homologue of the mammalian spinocervicothalamic system. A brief survey of the literature shows that such a system is much more common in vertebrates than previously thought.

INTRODUCTION

The presence of a bisynaptic spinocervicothalamic pathway composed of spinocervical and cervicothalamic tracts has been demonstrated for mammals (Morin, 1955; Nijensohn and Kerr, 1975; Boivie, 1983). The first physiological studies suggested that this pathway forms a rapidly conducting system carrying information from the skin to the cerebral cortex (Catalano and Lamarche, 1957). Subsequent tract-tracing studies in several mammalian species demonstrated the spinocervical tract as an ipsilateral projection from dorsal horn neurons throughout the spinal cord ascending via the dorsolateral funiculus to the lateral cervical nucleus (LCN). The LCN is a special group of neurons within the white matter just ventrolateral to the dorsal horn in the uppermost cervical segments (C1-C3). The projections from the LCN decussate and pass via the contralateral medial lemniscus towards mesencephalic and thalamic somatosensory areas and form the cervicomesencephalic and cervicothalamic tracts, respectively (see Willis and Coggeshall, 1991). So far, a definitive spinocervical tract has not been demonstrated in nonmammalian vertebrates. Only fragmentary data are available (e.g., Ebbesson, 1967; Finger, 1981; Forehand and Farel, 1982; Ito *et al.*, 1986; Necker, 1989; Ronan and Northcutt, 1990).

In anurans, the presence of a spinal lemniscus passing via the ventral brain stem and innervating the rhombencephalic and mesencephalic parts of the reticular formation in particular was demonstrated using anterograde degeneration techniques (Ebbesson, 1969, 1976; Hayle, 1973a,b). Recently, we demonstrated a distinct spinothalamic component in amphibians (A. Muñoz *et al.*, 1994a). Moreover, well-established ascending projections from a dorsal column nucleus present at the obex and upper spinal segments, via the medial lemniscus were shown for anurans (A. Muñoz *et al.*, 1993; 1994b; 1995a). The possible existence of an ascending pathway from spinal cells comparable to the LCN of mammals was considered briefly since injections of retrograde tracers into the thalamus or the mesencephalic torus semicircularis revealed a distinct cell population situated in the dorsolateral part of the spinal cord at upper cervical segments (A. Muñoz *et al.*, 1995a). The location, morphology and ascending projections of these cells suggested the presence of an anuran homologue of the LCN of mammals, and prompted the present study. A separate nucleus in the dorsolateral part of the upper cervical spinal cord or at the obex is not distinguishable as a cytoarchitectonic entity (Ebbesson, 1976; Nikundiwe and Nieuwenhuys, 1983).

The aim of the present study is to characterize this cell group in the lateral aspect of the spinal dorsal horn by studying its afferent and efferent connections. The powerful and fast tracer low-weight (3kD) biotinylated dextran amine (BDA) was used to label ascending spinal projections, to trace possible LCN projections retrogradely from the ventral thalamus and torus semicircularis (the mesencephalic somatosensory target in anurans), and to analyze the cells of origin of the possible spinocervical tract. BDA can be used for anterograde as well as retrograde tracing (Fritzsch, 1993; A. Muñoz *et al.*, 1995a). An *in vitro* approach

was used in the clawed toad, *Xenopus laevis:* an isolated preparation of the central nervous system (CNS) well suited for a variety of neuroanatomical tracing techniques (Luksch *et al.*, 1995). Part of this study was published in abstract form (A. Muñoz *et al.*, 1995b).

MATERIALS AND METHODS

In the present study a total of 25 young adult *Xenopus laevis* were used in tracing experiments under *in vitro* conditions (Luksch *et al.*, 1995; based on Cochran *et al.*, 1987). The animals were deeply anesthetized with a 0.2% solution of MS222 and cooled to a body temperature of about 5°C. The heart was exposed by rapid thoracotomy in order to perfuse the animal transcardially with approximately 40 ml iced Ringer's solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, 11 mM glucose) that had been oxygenized with carbogen (95% O_2, 5% CO_2) to a pH of 7.3 (Straka and Dieringer, 1993). The brain and spinal cord were isolated by a dorsal approach by removing the overlaying bony tissue of the skull and vertebral column. After isolation, the CNS was tranferred into a dish with fresh iced Ringer. Subsequently, the dura mater and the choroid plexus were removed to facilitate oxygen diffusion into the tissue. Applications of BDA (3kD, Molecular Probes D-7135), recrystallized at the tip of glass micropipettes or sharp tungsten needles, were made at the dorsal horn of cervical, thoracic or lumbar spinal levels (10 cases), the ventral thalamus (5 cases) and the torus semicircularis (5 cases). In the latter cases, the CNS was cut transversally at midthalamic and midmesencephalic levels, respectively. In addition, small applications of 3kD BDA were made into the dorsolateral funiculus at cervical (2 cases) and obex (3 cases) levels. The brains were kept for 15-18 hours at about 15°C in continuously oxygenated Ringer's solution with carbogen. They were then immersed for 3-5h in 4% paraformaldehyde in phosphate buffer 0.1 M, pH 7.4 (PB), embedded in polyacrylamide (see ten Donkelaar and de Boer-van Huizen, 1991), and cryoprotected overnight in a 15% sucrose solution in PB. Sections were cut transversally at 40 µm on a freezing microtome and collected in 0.05 M Tris buffer (pH 7.6). For visualizing BDA, an avidine biotin complex (Vectastain ABC Elite Kit, Vector Laboratories) was used, followed by heavy metal intensification of the diaminobenzidine (DAB)-based peroxidase reaction product (Adams, 1981). Selected sections were counterstained with 1% cresyl violet solution. The sections were mounted on gelatin coated glasses and coverslipped with glycerin-gelatin.

The nomenclature used is based on studies by Ebbesson (1976) on the subdivision of the spinal grey and by Nikundiwe and Nieuwenhuys (1983) on brainstem structures.

RESULTS

In the present study, BDA was applied to the dorsal part of lumbar (Fig.1), thoracic (Fig. 2) and cervical spinal segments. As a general feature, after tracer application to the spinal cord, distinct ascending fiber tracts were noted in the dorsal funiculus and in the dorsolateral funiculus, ipsilaterally, and mainly contralaterally in the ventral and ventrolateral funiculi. The course of the contralaterally ascending pathway coincides with that observed in previous studies (Ebbesson, 1976; M. Muñoz *et al.*, 1991; A. Muñoz *et al.*, 1994a). Its main targets and cells of origin will be discussed in a companion paper (A. Muñoz *et al.*, in preparation). Here, only the ascending fibers passing

via the dorsal and the dorsolateral funiculi will be discussed. In all experiments, a small contralateral component of ascending fibers in the dorsolateral funiculus was labeled. In a second set of experiments, BDA was used as a retrograde tracer to identify the possible anuran homologue of the mammalian lateral cervical nucleus (LCN). BDA was applied to the torus semicircularis (Fig. 4) and the thalamus (Fig. 5). Finally, the cells of origin of the possible spinocervical tract were studied after small BDA applications to the dorsolateral funiculus at the rostral spinal cord (Fig. 6).

Lumbar spinal cord applications

In this set of experiments, BDA was placed unilaterally into the dorsal spinal cord. The application sites included the dorsal horn, but also the dorsal and dorsolateral funiculi (Fig. 1). The applications were made at the lumbar level between dorsal roots 9 and 10. Both descending and ascending projections were observed. Rostral to the application, anterogradely labeled fibers were observed in the ipsilateral dorsal and dorsolateral funiculi. The fibers in the dorsolateral funiculus give off thin collaterals that innervate the lateral aspect of the grey in the deep part of the dorsal field and in the lateral field between the levels of the spinal dorsal roots 9 and 5. The more rostral part of the spinal grey is not innervated by lumbar dorsolateral funiculus fibers. Fibers from the dorsal funiculus reach the superficial part of the dorsal field, while others course more ventrally into the lateral field and even the ventral fields. This pattern of innervation is present up to the 5th spinal segment. A few scattered fibers cross to innervate the contralateral superficial part of the dorsal field. Rostrally, the ascending fibers form a compact bundle in the medial part of the dorsal funiculus, and just a few fibers turn laterally and enter the grey matter of the rostral spinal segments.

At upper cervical segments and at the level of the obex, most of the labeled fibers in the dorsolateral funiculus turn dorsomedially and massively innervate the neurons in the dorsolateral grey (Figs. 1, 3A). At these levels, the fibers that course in the dorsal funiculus massively innervate the medial portion of the ipsilateral dorsal column nucleus (DCN) and less densely the caudal aspect of the nucleus of the solitary tract. Only a few fibers terminate in the contralateral DCN. Just caudal to the obex a band-shaped area is present in the grey where the projections from the dorsal and dorsolateral funiculi overlap (Figs. 1, 3A). This band marks the ventral border of an area known to be occupied by fibers of the descending trigeminal tract and the cells related to them (González *et al.*, 1993).

Thoracic spinal cord applications

After tracer applications to the dorsal part of the spinal cord at various thoracic levels, ascending and descending projections were observed (Fig. 2). Caudal to the application site, labeled fibers course in the dorsal funiculus and, especially, in the dorsolateral funiculus and innervate the dorsal field and, to a lesser extent, the lateral and ventral fields up to the lumbar segments. Rostral to the BDA application the labeling of ascending fibers prevails in the dorsolateral funiculus. The innervation of the deep part of the dorsal field continues up to the level of the DCN. At the obex level and slightly caudally, the labeled fibers in the dorsal funiculus innervate the ipsilateral DCN, with a minor contralateral component, while the fibers coursing in the dorsolateral funiculus terminate around a group of neurons along the ventrolateral border of the dorsal horn in the two first spinal segments (Figs. 2,

3B). At these levels, the terminal fields of both systems form a band-shaped area with an overlapping zone where terminals from fibers in the dorsal and dorsolateral funiculi intermingle.

Cervical spinal cord applications

Following tracer application to the dorsal part of the cervical spinal cord, a similar pattern of labeled fibers as described above for the thoracic and lumbar cases was observed. When the BDA application site was restricted to the dorsal and lateral fields of the spinal grey in cervical segments 3-4, only a few labeled fibers passing caudalwards via the dorsal funiculus were noted, whereas labeled fibers in the dorsolateral funiculus could be observed up to lower lumbar spinal segments, innervating the dorsal and lateral fields throughout their course. Rostral to the application site, numerous labeled fibers were found in the dorsolateral funiculus and in the lateral part of the dorsal funiculus. Fibers from both funiculi innervate the dorsal field of the spinal grey at cervical segments. More rostrally, the fibers from the dorsal funiculus innervate the lateral aspect of the DCN, whereas the fibers in the dorsolateral funiculus innervate the group of neurons along the ventrolateral border of the dorsal horn (Fig. 3C). This innervation zone overlaps with the innervation of the DCN.

Thalamic and toral BDA applications

Experiments with BDA application to the ventral thalamus or the torus semicircularis in the mesencephalon were used to study whether the particular group of neurons along the ventrolateral border of the dorsal horn in rostral spinal segments gives rise to ascending projections to the mesencephalon and the thalamus. In a previous study (A. Muñoz *et al.*, 1995a) tracer applications that included the ventral thalamus resulted in retrogradely labeled cells in what was identified as the DCN. However, the most ventrolaterally located cells of this population extend their dendrites laterally and were seen to be more closely related with the dorsolateral funiculus than with the dorsal funiculus. A similar pattern was observed when the tracer was applied to the lateral aspect of the torus semicircularis. The *in vitro* experiments illustrated in figures 4 and 5 clearly demonstrate that the cell population previously labeled as dorsal column nucleus, in fact, is composed of two more or less separate cell groups: the medial DCN and a more lateral cell group in the area where fibers passing via the dorsolateral funiculus massively terminate. Due to its position and connections, the name *lateral cervical nucleus* (LCN) will be introduced for this cell group. It should be emphasized that in anurans this cell group is not recognizable as a cytoarchitectonic entity (see Fig. 3D). This neuronal population extends slightly more caudally than the DCN, i.e. from the obex level up to the second cervical segment. The cells in the LCN project to the torus semicircularis and the thalamus via the medial lemniscus (Figs. 4, 5). The cells in the LCN occupy a position in the lateral aspect of the spinal grey and their dendrites extend profusely into the dorsolateral funiculus (Figs. 4, 5). Only a few scattered labeled cell bodies were observed within the dorsolateral funiculus itself. The fibers ascending in the dorsolateral funiculus richly innervate the area of the LCN.

Tracer applications into the cervical part of the dorsolateral funiculus

In order to identify the cells of origin of the fibers ascending in the dorsolateral funiculus towards the lateral cervical nucleus, small applications of BDA

were made to the dorsolateral funiculus at the obex level (Fig. 6A) and at the second cervical spinal segment. By means of retrograde tracing, the cells of origin of this spinocervical tract were demonstrated. A large population of cells was labeled, mainly ipsilaterally to the application site, although a small number of contralateral cells were present in the ventral fields of the grey at thoracic and lower cervical segments (Fig. 6A). The ipsilateral population is predominantly located in the dorsal horn from cervical (Fig. 6B,C) to sacral segments. The majority of the cells are found in the deeper part of the dorsal field of the spinal grey. More sparsely distributed neurons are present in the superficial part of the dorsal horn. Most of the labeled neurons have round-to-oval somata with dorsally or ventrolaterally directed processes that often enter the dorsolateral funiculus (Fig. 6C). Larger, triangular or irregular cells are more rarely labeled. A small number of cells are ipsilaterally located in the lateral spinal field at thoracic levels.

DISCUSSION

In the present study the organization of the ascending projections in the dorsal and dorsolateral funiculi was studied in the clawed toad, *Xenopus laevis*. In particular, the presence of a spinocervical tract and of a possible anuran homologue of the mammalian lateral cervical nucleus were investigated.

The relatively new tracer BDA was used in an *in vitro* approach. In previous studies the suitability of isolated preparations of the anuran central nervous system has been discussed (Luksch *et al.* 1995; A. Muñoz *et al.* 1995a). BDA was first described to be very successfully transported anterogradely by neuron processes (Veenman *et al.*, 1992). However, BDA can also be used effectively as a retrograde tracer (A. Muñoz *et al.*, 1995a). When BDA is applied iontophoretically, it will be transported primarily anterogradely. In contrast, application in dry form, both *in vivo* and *in vitro*, results in an effective retrograde transport. The present study shows that BDA, when applied as dry crystals, is also an effective anterograde tracer. It should be emphasized, however, that even the smallest BDA application to the spinal cord also results in retrograde labeling of fibers and cells.

Tracer applications to the dorsal part of the spinal cord at cervical, thoracic and lumbar levels, once more demonstrated a somatotopical arrangement of the fibers ascending in the dorsal funiculus including their pattern of termination in the DCN as previously noted in anurans (Antal *et al.*, 1980; Nikundiwe *et al.*, 1982; M.Muñoz *et al.*, 1991; A.Muñoz *et al.*, 1995a). These fibers include primary afferents as well as second-order projections towards the DCN, i.e. the postsynaptic dorsal column system (ten Donkelaar and de Boer van Huizen, 1991; A.Muñoz *et al.*, 1995a).

A well-developed system of ascending fibers in the dorsolateral funiculus was demonstrated. The targets of these fibers include the dorsal and lateral spinal fields of the grey and, especially, the area of the lateral cervical nucleus. Similar observations were made in the Spanish green frog, *Rana perezi* (A. Muñoz *et al.* 1995b). Other brainstem projections will be discussed in a companion paper (A. Muñoz, in preparation).

In experiments where the tracer application site included the dorsal and lateral spinal fields as well as the dorsolateral funiculus some fibers were found leaving that funiculus to innervate the dorsal and lateral spinal fields rostral and caudal to the spinal

segment involved. Some of these fibers may be spinal primary afferents running in Lissauer's tract (Antal *et al.*, 1980; Nikundiwe *et al.*, 1982; M.Muñoz *et al.*, 1991; A.Muñoz *et al.*, 1995a). However, also non-primary intersegmental intraspinal projections through the dorsolateral funiculus may exist in amphibians, as is the case in mammals in which the spinocervical tract neurons give off collateral branches to various targets of the spinal grey at different spinal levels (Snow *et al.*, 1976; Brown *et al.*, 1977; Rastad et al, 1977; Jankowska *et al.*, 1979; Maxwell and Koerber, 1986; Cao *et al.*, 1993). Some of the dorsolateral funiculus fibers that innervate the different spinal fields caudal to the application site may belong to descending projections from different spinal and supraspinal sources, including the LCN and the lateral reticular formation. In these structures, retrogradely labeled neurons were observed. Close to the obex, the dorsolateral funiculus massively gives off thin fibers, directed dorsomedially towards a region located along the ventral border of the dorsal horn in rostral spinal segments. This zone represents the anuran homologue of the mammalian LCN.

The tract-tracing experiments presented in this study suggest that the spinal projections to the LCN arise in cells located throughout the spinal cord, mainly in the ipsilateral deep dorsal field. The spinocervical tract in mammals is an excitatory glutamatergic tract (Broman *et al.*, 1990; Kechagias and Broman, 1994) known to arise in spinal cells that receive an input from the periphery (see Willis and Cogheshall, 1991). The distribution of these spinal cells was studied in the rat, cat and dog (Baker and Giesler, 1984; Craig, 1976, 1978; Craig *et al.*, 1992). They are distributed predominantly in the ipsilateral nucleus proprius, substantia gelatinosa and lamina IV, with fewer cells in lamina V. At cervical levels, scattered cells in laminae I, VI and VII also contribute to the spinocervical tract. A small contralateral component from laminae I, VII and VIII was described in cats (Brown *et al.*, 1980). A possible spinocervical tract was also demonstrated in other non-mammalian vertebrates (see Table I). In birds, van den Akker (1970) showed a "dorsolateral ascending bundle" in the dorsolateral funiculus that arises in neurons found in the deep part of the dorsal horn. At cervical levels, this bundle innervates the deep dorsal and central spinal grey. More recent tract-tracing studies showed various ascending non-primary spinal projections in the dorsolateral funiculus of the pigeon (Funke and Necker, 1986; Funke 1988; Necker, 1991), most likely including the spinocervical tract. The ascending spinal projections through the dorsolateral funiculus in birds arise from neurons located in laminae I, IV, V and in Clarke's column (Funke and Necker, 1986; Funke 1988; Necker, 1991). In reptiles, the only evidence for the presence of a spinocervical tract comes from an anterograde degeneration study in the tegu lizard, *Tupinambis teguixin*. Ebbesson (1967) noted that at caudal brainstem levels some collateral fibers leave the dorsolateral funiculus and innervate an area located dorsal to the hypoglossal nucleus and ventral to the DCN. In other anamniotes, evidence for a spinocervical tract projecting to a lateral cervical nucleus is at least suggestive. In agnathans (Northcutt and Ebbesson, 1980; Ronan and Northcutt, 1990) as well as in cartilaginous (Hayle, 1973a,b; Ebbesson and Hodde, 1981; Smeets *et al.*, 1984) and bony fishes (Hayle 1973a,b; Finger, 1981), ascending spinal projections were demonstrated via the dorsal part of the lateral funiculus. No separate site of termination, reminiscent of an LCN, was noted.

The mammalian LCN extends from the obex level to the second spinal segment, and is formed by

neurons close to and extending into the dorsolateral funiculus. Their axons pass via the medial lemniscus, and mainly reach the mesencephalic somatosensory intercollicular zone and the ventrobasal complex of the thalamus, and form the cervicomesencephalic and cervicothalamic tracts, respectively (Willis and Coggeshall, 1991). There is substantial evidence for the presence of an LCN in non-mammalian vertebrates (Table I). In birds, the available data on the existence of the spinocervicothalamic pathway and the LCN are sparse. With a silver impregnation technique, Ramón y Cajal (1911) already noted an interstitial nucleus in the chick embryo, formed by triangular cells located throughout the spinal dorsolateral funiculus, but preferentially at cervical levels. In the pigeon, the LCN was defined as a cytoarchitectonic entity at upper cervical levels (Karten and Hodos, 1967). However, van den Akker (1970) could not distinguish an LCN, although he found some cells located within the dorsolateral funiculus at high spinal levels. Also in the pigeon, although more caudally, Necker (1990) described a lateral spinal nucleus in the dorsolateral funiculus at the level of the cervical intumescence. Necker (1989) noted some neurons located in the lateral neck of the dorsal horn close to the dorsolateral funiculus in the first cervical segment that were retrogradely labeled from the contralateral thalamus. This cell population is located in a position comparable to that of the mammalian (see Willis and Coggeshall, 1991) and amphibian (present study) thalamic and midbrain projecting LCN neurons. It may represent the origin of the cervicothalamic tract in birds. In reptiles, an LCN has not been described as a cytoarchitectonic entity (Ebbesson 1967, 1969; Kusuma, 1979; Künzle and Woodson, 1982; Pritz and Stritzel, 1986). Retrograde tracer studies (Hoogland, 1981, 1982; Pritz and Stritzel, 1989, 1990) did not focus on the possible presence of an LCN.

In anurans, no separate LCN was noted in Nissl (Opdam *et al.*, 1976; Nikundiwe and Nieuwenhuys, 1983; see also Fig. 3D) or Golgi (Ebbesson, 1976) studies, but even the anuran DCN was not clearly defined until recently (A. Muñoz *et al.*, 1995a). The existence of an anuran LCN was suggested in a developmental study (Forehand and Farel, 1982). In *Rana catesbeiana* tadpoles, HRP was applied to the lateral aspect of the reticular formation at rhombencephalic levels between the Vth and the Xth nerves and to the tectum. Some contralateral neurons were retrogradely labeled at cervical levels in the marginal zone, just outside the intermediate grey. Moreover, in a recent study focussed on the anuran medial lemniscus (A. Muñoz *et al.*, 1995a) it was noted that some neurons, then called the ventrolateral component of the DCN, were retrogradely labeled from the ventral thalamus and, more conspicuously, from the lateral aspect of the torus semicircularis. These cells are intermingled with the proper DCN neurons in *Xenopus laevis*, but somewhat more segregated in *Rana perezi* (A. Muñoz *et al.*, 1995b), and extend more caudally than the DCN, up to the second spinal segment. This group of cells represents the anuran homologue of the LCN in mammals. Although only a few cells are actually present in the dorsolateral funiculus itself, the dendrites of the LCN neurons are mainly directed ventrolaterally and extend throughout the dorsolateral funiculus. Nevertheless, at the obex level, where the DCN and the LCN coexist, it is difficult to define neurons at intermediate locations as belonging to the DCN or the LCN. Only the morphology of their dendrites, mainly oriented dorsally or ventrolaterally to the dorsal funiculus or the dorsolateral funiculus, respectively, and the more rostral extent of the DCN, allow to distinguish the neurons of the DCN and the LCN.

In other anamniotes, data on the presence of an LCN are sparse (Table I). In teleosts, a comparable nucleus was observed at the obex level and the first cervical spinal segments (Finger, 1981; Ito *et al.*, 1986). In *Sebasticus marmoratus*, Ito *et al.* (1986) demonstrated that an LCN projects to the ventromedial thalamic nucleus. Additionally, in lampreys, experiments with diencephalic or mesencephalic HRP applications resulted in retrogradely labeled neurons at the obex level and in the first cervical spinal segments suggesting the presence of a putative LCN in agnathans (Ronan and Northcutt, 1990).

Broman (1994) reviewed the chemoarchitecture of the area of the mammalian LCN. In short, GABA-, catecholamine- (see Doyle and Maxwell, 1994), serotonin- and substance P- positive terminals were found within the LCN. The GABA-positive terminals are thought to belong to intrinsic LCN neurons, but the substance P-positive and serotonergic innervation of the LCN is thought to have a spinal and supraspinal origin, respectively. Glutamatergic terminals within the LCN are thought to belong to spinocervical tract neurons and also to cervicothalamic tract collateral axons that terminate within the LCN itself (Broman, 1994). Within the limits of the anuran LCN, GABA-, glycine- and parvalbumin-positive cells are present, as well as terminals immunopositive for CGRP, substance P, neuropeptide Y and serotonin (A. Muñoz *et al.* 1995a). Additionally in anurans, processes of catecholaminergic neurons in the vicinity of the nucleus of the solitary tract extend to the area where LCN neurons are located (González and Smeets, 1994; A. Muñoz *et al.*, 1995a).

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TABLE I

The distribution of a spinocervicothalamic system in vertebrates

Vertebrate group	Spinocervical tract	Lateral cervical nucleus	Cervico-mesencephalic tract	Cervico-thalamic tract
Agnathans				
Lampreys				
• Silver lamprey *(Ichthyomozon unicuspis)*	*[21]	?	?	?
• Sea lamprey *(Petromyzon marinus)*	*[20,21]	+[21]	+[21]	+[21]
Hagfishes				
• Pacific hagfish *(Eptatretus stouti)*	*[21]	?	?	?
Gnathostomes				
Cartilaginous fishes				
• Spotted dogfish *(Scyliorhinus canicula)*	*[7]	–[22]	?	?
• Nurse shark *(Ginglymostoma cirratum)*	*[2]	?	?	?
Bony fishes				
• Sea robin *(Prionotus carolinus)*	+[3]	?	?	?
• *Sebasticus marmoratus*	?	+[10]	?	+[10]
• Rudd *(Scardinius erythophthalmus)*	*[7,8]	?	?	?
Amphibians				
• Tiger salamander *(Ambystoma tigrinum)*	+[9]	?	?	?
• Ribbed newt *(Pleurodeles waltl)*	+[16]	+[16]	+[16]	+[16]
• Clawed toad *(Xenopus laevis)*	+[15]	+[15]	+[15]	+[15]
• Bullfrog *(Rana catesbeiana)*	?	+[4]	+[4]	?
• Large green frog *(Rana perezi)*	+[14]	+[13,14]	+[14]	+[14]
Reptiles				
• Red-eared turtle *(Pseudemys scripta elegans)*	*[12]	?	?	*[12]
• Tegu lizard *(Tupinambis teguixin)*	+[1]	+[1]	?	?
Birds				
• Pigeon *(Columba livia)*	+[5,6,19,23]	+[11,17]	?	+[18]
Mammals				
• Rodents, carnivores, primates	+[24]	+[24]	+[24]	+[24]

Symbols used: + present; - not reported or present; * indirect or suggestive evidence; ? unknown.
References: 1 - Ebbesson (1967); 2 - Ebbesson and Hodde (1981); 3 - Finger (1981); 4 - Forehand and Farel (1982); 5 - Funke and Necker (1986); 6 - Funke (1988); 7 - Hayle (1977a); 8 - Hayle (1973b); 9 - Herrick (1930); 10 - Ito et al. (1986); 11 - Karten and Hodos (1967); 12 - Künzle and Woodson (1982); 13 - A. Muñoz *et al.* (1995a); 14 - A. Muñoz *et al.* (1995b); 15 - A. Muñoz *et al.* (present study); 16 - A. Muñoz *et al.* (unpublished observations); 17 - Necker (1989); 18 - Necker (1990); 19 - Necker (1991); 20 - Northcutt and Ebbesson (1980); 21 - Ronan and Northcutt (1990); 22 - Smeets *et al.* (1984); 23 - van den Akker (1970); 24 - Willis and Coggeshall (1990).

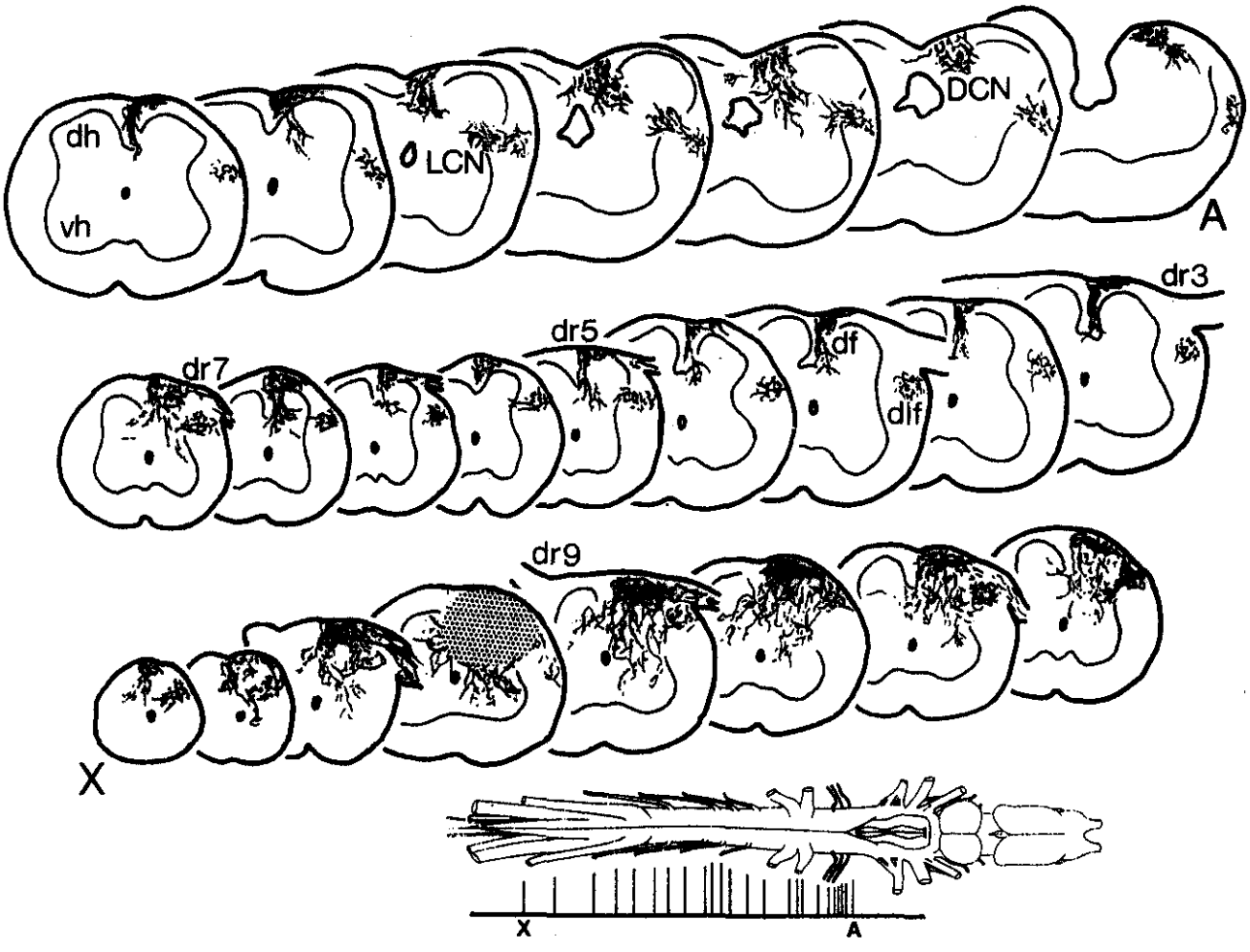


Fig. 1. Schematic drawing of transverse sections of the brain stem and spinal cord of a young adult *Xenopus laevis* showing the labeling in the dorsal and dorsolateral funiculi after *in vitro* application of 3kD BDA into the lumbar spinal cord, between the 9th and 10th dorsal roots. The level of the sections in this and other figures is indicated along a dorsal view of the central nervous system.

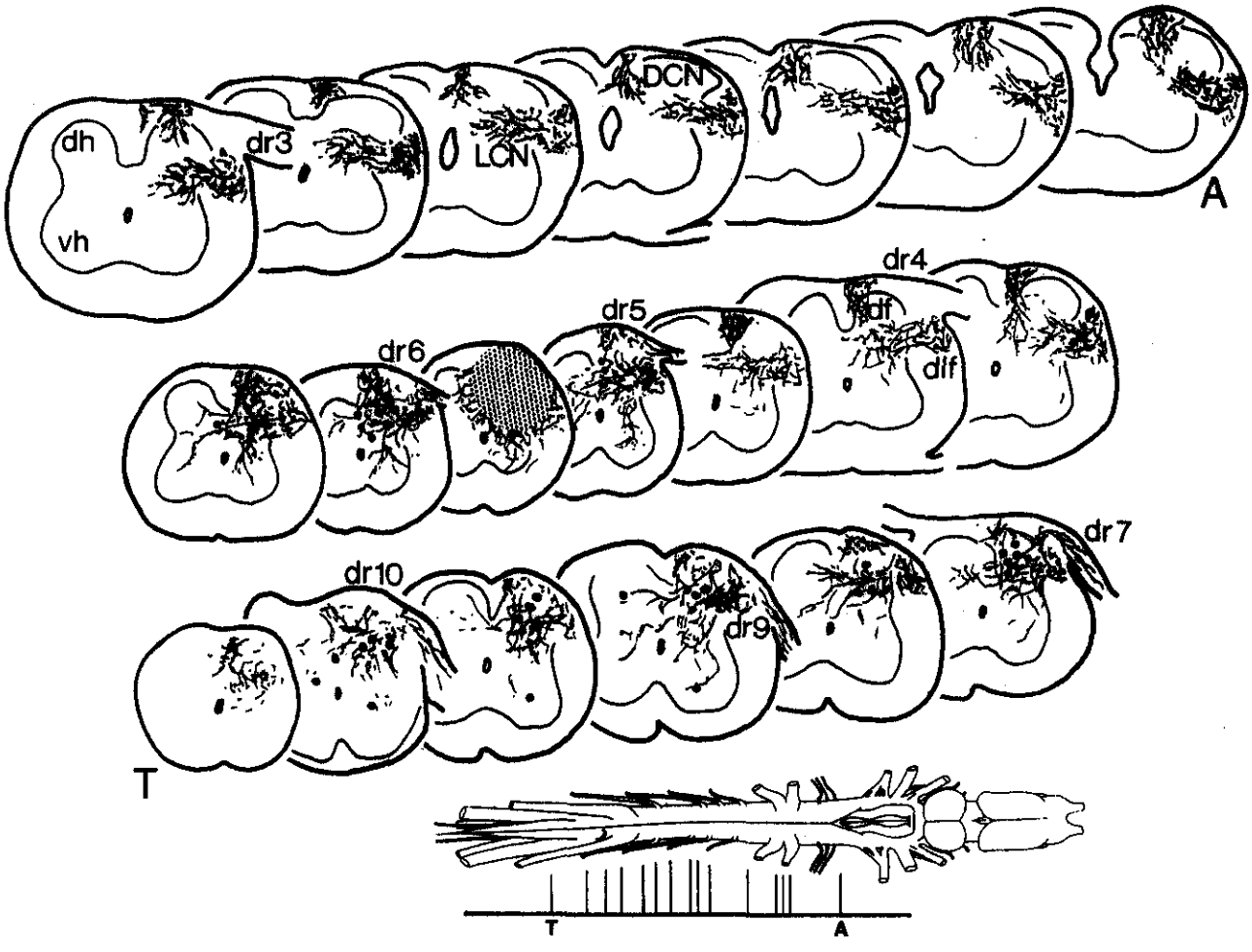


Fig. 2: Schematic drawing of transverse sections of the brain stem and the spinal cord of a young adult *Xenopus laevis* showing the labeling in the dorsal and dorsolateral funiculi after *in vitro* 3kD BDA application into the thoracic spinal cord between the 5th and 6th dorsal roots.

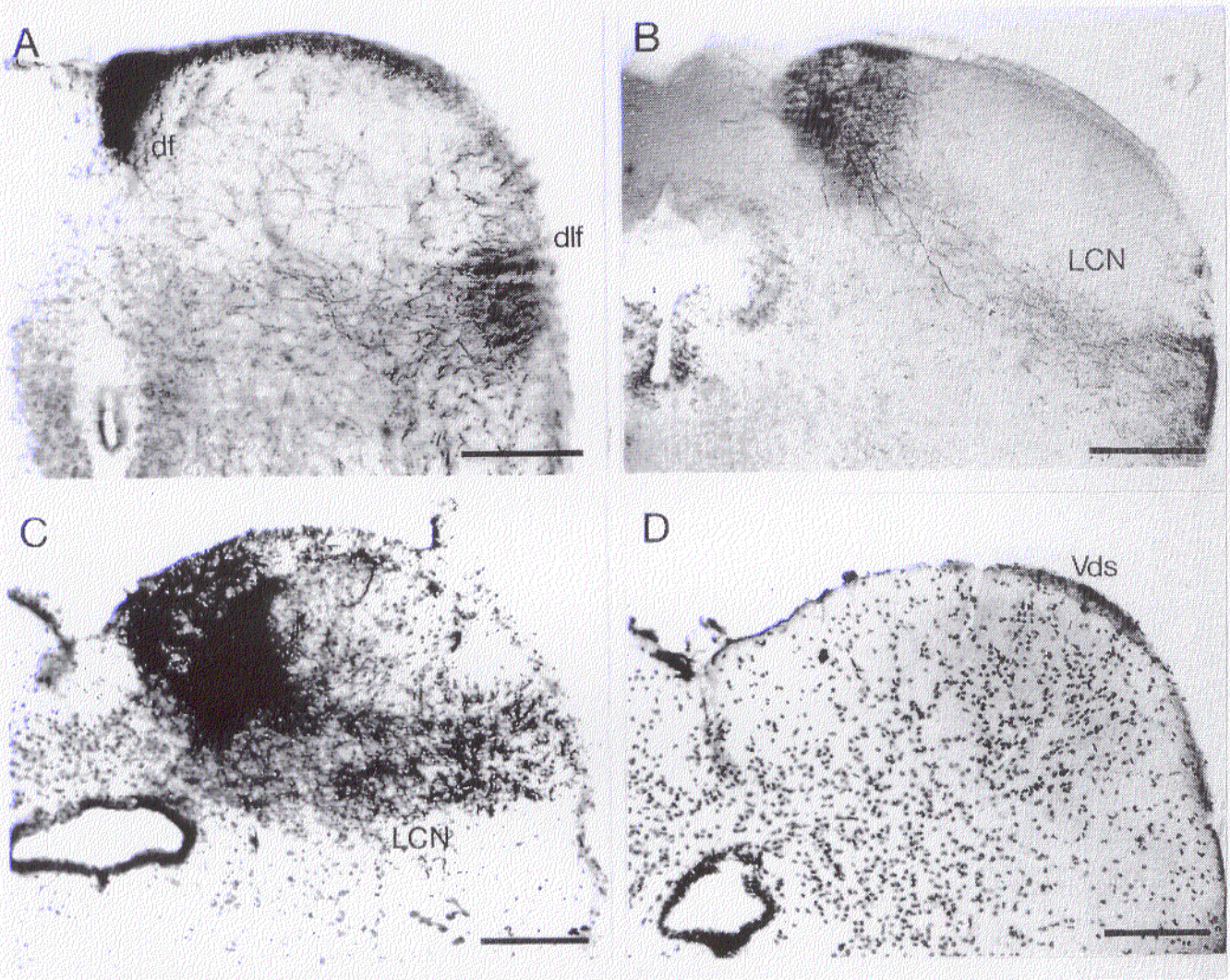


Fig. 3: Photomicrographs showing anterogradely labeled fibers in the dorsal column nucleus and in the lateral cervical nucleus (A-C), and a Nissl-stained section at the same level. A, BDA-labeling after lumbar application; B, BDA-labeling after a thoracic application; C, BDA-labeling after a cervical application; D, Nissl-stained section. Note the presence of a distinct nucleus of the descending tract of the trigeminal nerve in the lateral corner. The dorsal column and lateral cervical nuclei are ill-defined. Scale bars indicate 100 µm.

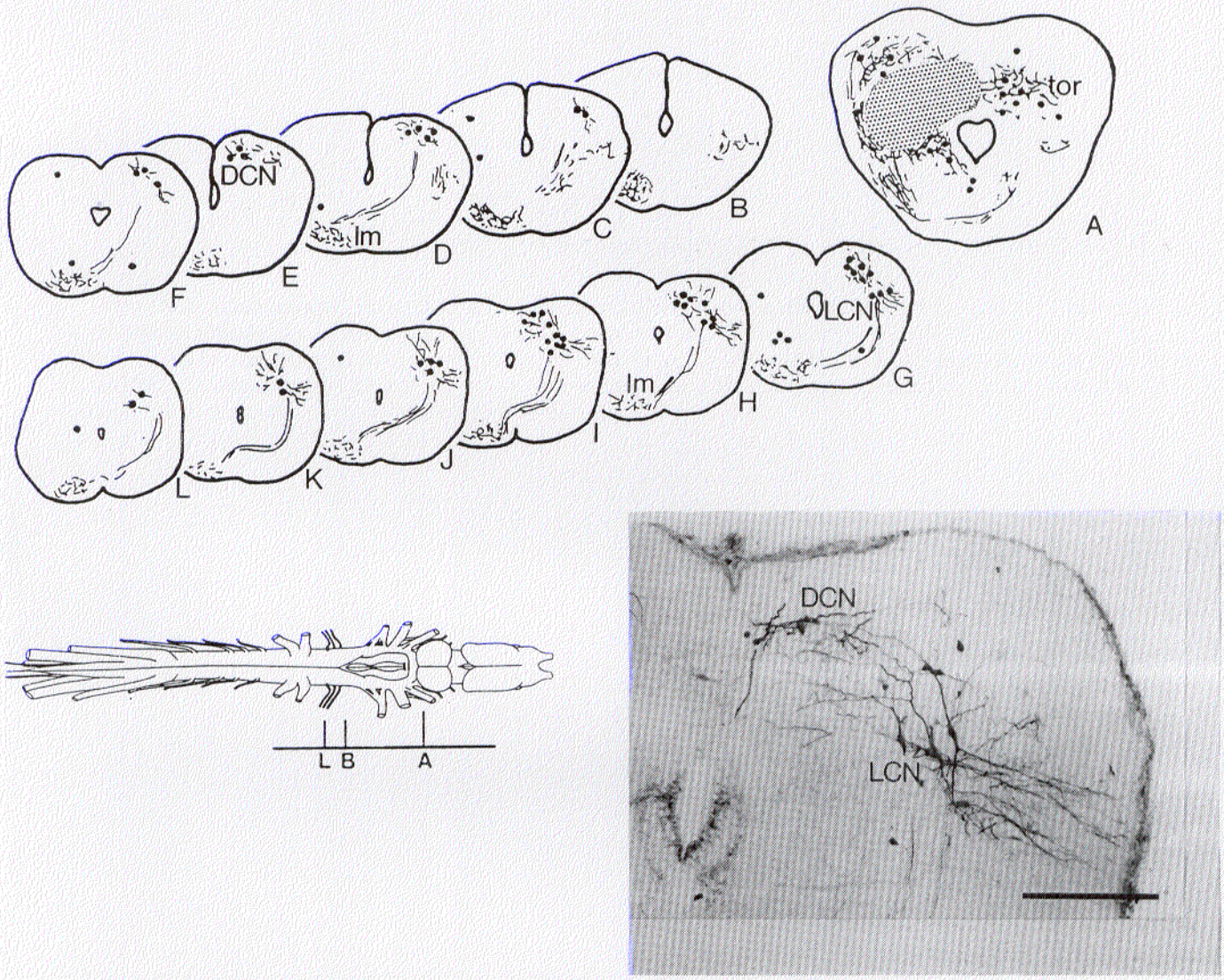
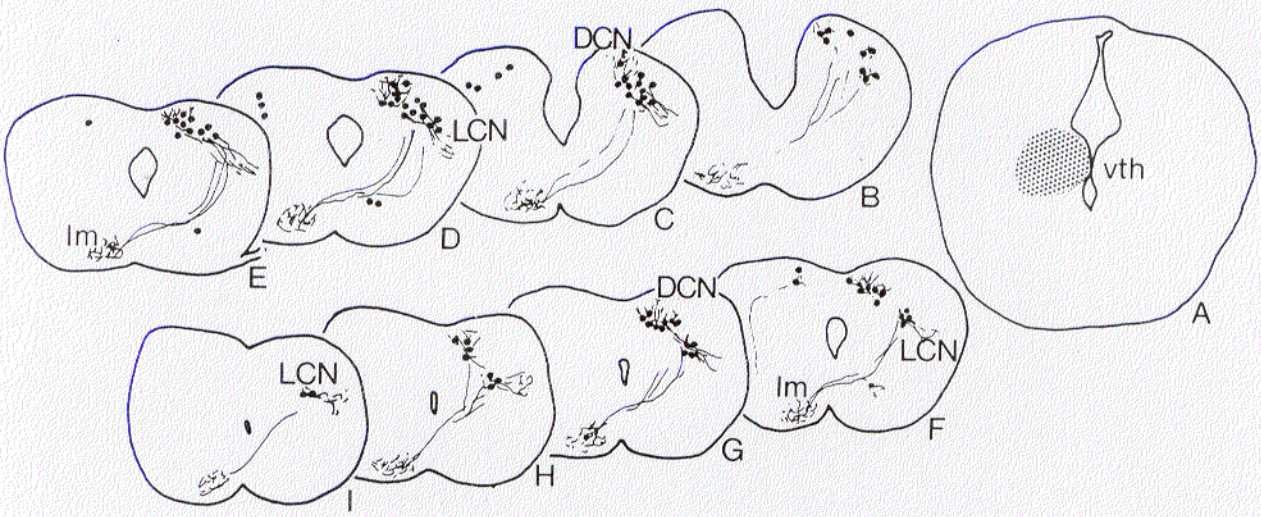
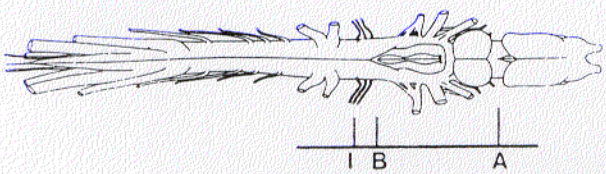


Fig. 4: Schematic drawing illustrating the distribution of retrogradely labeled neurons in the caudal part of the rhombencephalon and in the rostral part of the spinal cord of *Xenopus laevis*. BDA was applied to the torus semicircularis. Inset shows an example of the labeling in the dorsal column nucleus and in the lateral cervical nucleus. LCN neurons possess dorsally oriented dendrites as well as ventrolaterally directed dendrites aimed at the dorsolateral funiculus. Scale bar indicates 100 µm.





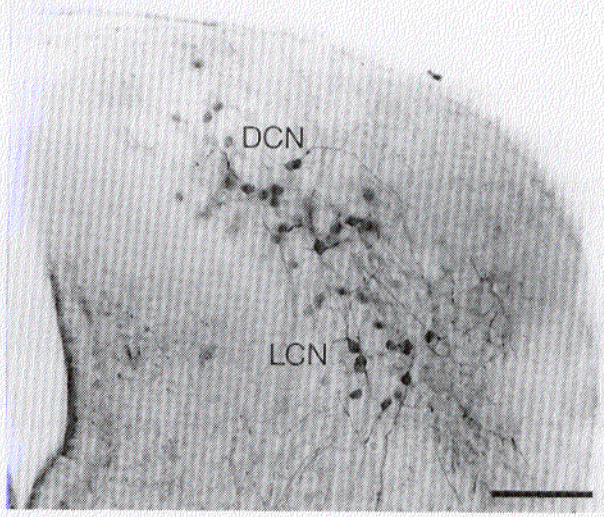


Fig.5: Schematic drawing illustrating the distribution of retrogradely labeled neurons in the caudal part of the rhombencephalon and in the rostral part of the spinal cord of *Xenopus laevis*. BDA was applied to the ventral thalamus. Inset shows an example of the labeling in the dorsal column nucleus and in the lateral cervical nucleus. Scale bar indicates 100 µm.

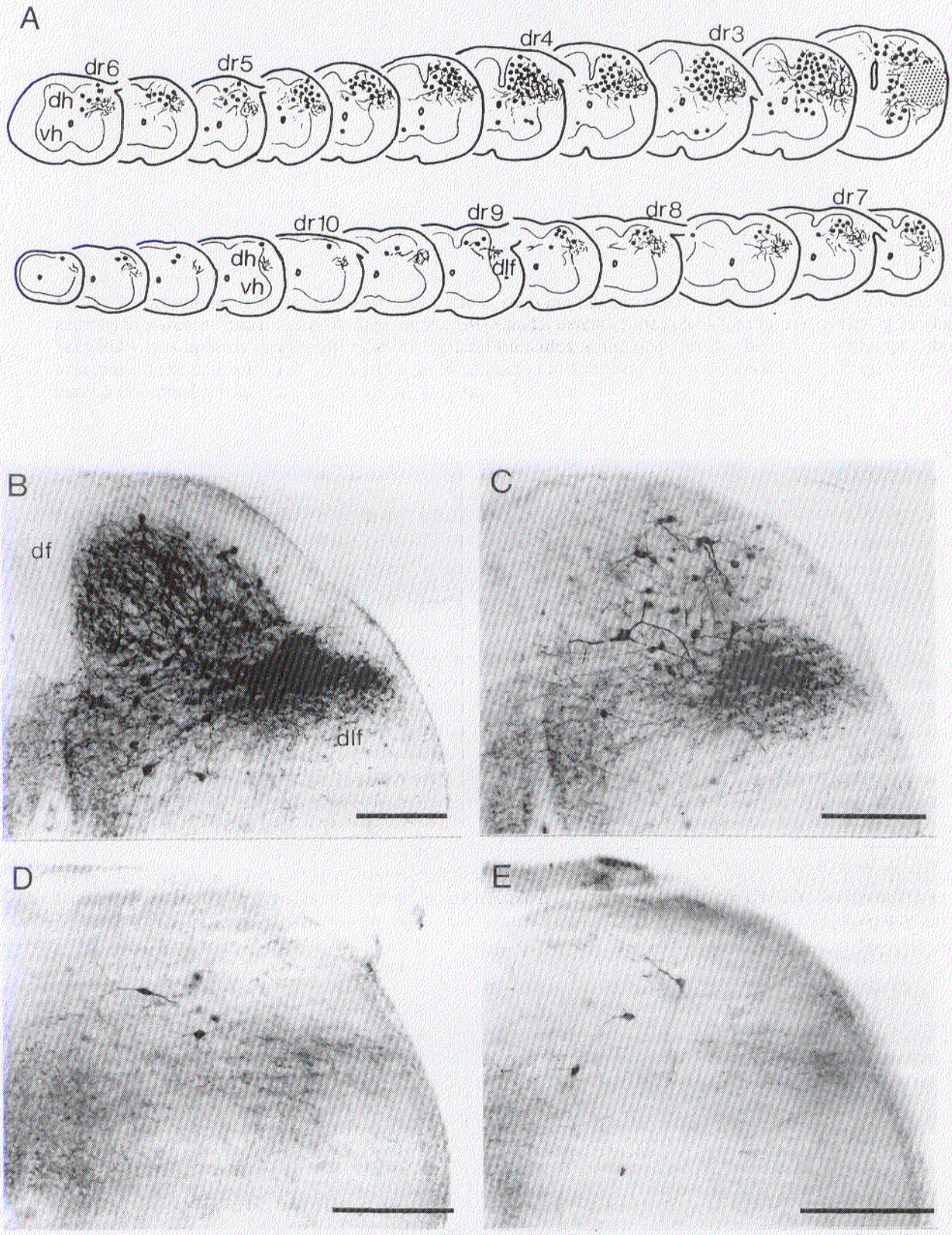


Fig. 6: A, Schematic drawing of transverse sections showing labeled fibers in the dorsolateral funiculus and neurons in different spinal fields throughout the rostrocaudal extent of the spinal cord in an experiment in which 3kD BDA was applied *in vitro* into the dorsolateral funiculus at the obex level. The photomicrographs show examples of the cells of origin of the spinocervical tract at midcervical (B), low cervical (C), thoracic (D) and lumbar (E) spinal levels. Scale bars indicate 100 µm.

ABBREVIATIONS

DCN	dorsal column nucleus
df	dorsal funiculus
dh	dorsal horn
dlf	dorsolateral funiculus
dr(3-10)	third-tenth dorsal root
LCN	lateral cervical nucleus
lm	lemniscus medialis
tor	torus semicircularis
Vds	nucleus of the descending tract of the trigeminal nerve
vh	ventral horn
vth	ventral thalamus

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The dorsal column-medial lemniscal projection of anuran amphibians

The anuran dorsal column nucleus: Organization, immunohistochemical characterization, and fiber connections in Rana perezi and Xenopus laevis

Evidence for an anuran homologue of the mammalian spinocervicothalamic system: An in vitro tract-tracing study in Xenopus laevis

COMENTARIOS

5.4

En vertebrados terrestres básicamente existen dos sistemas de proyecciones espinales ascendentes (Willis y Coggeshall, 1991): 1) Sistema columna dorsal-lemnisco medial formado por proyecciones espinales aferentes primarias y no primarias que, a través del funículo dorsal, llegan hasta los núcleos de la columna dorsal, los cuales dan origen a la vía del lemnisco medial que asciende hasta el tálamo. 2) Sistema anterolateral formado por proyecciones aferentes secundarias que, a través del funículo ventrolateral, ascienden para alcanzar la formación reticular, el techo mesencefálico y el tálamo.

Igualmente se ha descrito la presencia de una tercera vía bisináptica denominada sistema espino-cervico-talámico, formado por el tracto espinocervical que termina en el núcleo cervical lateral (LCN), el cual proyecta contralateralmente, a través del lemnisco medial, hacia regiones somatosensoriales mesencefálicas y talámicas, originando los tractos cervicomesencefálico y cervicotalámico respectivamente (Willis y Coggeshall, 1991).

En el presente capítulo se presentan tres artículos en los que se ha estudiado la posible presencia de estos sistemas en anfibios. En el segundo, se ha logrado diferenciar, mediante la utilización de técnicas histoquímicas e inmunohistoquímicas, los distintos componentes celulares de la placa alar de la región del óbex que se encuentran pobremente diferenciados citoarquitectónicamente.

Citoarquitectura

En la mayoría de los estudios citoarquitectónicos del tronco cerebral en los anuros no se distinguió el núcleo de la columna dorsal (DCN) o del funículo dorsal (Ariëns Kappers y Hammer, 1918; Zeehandelaar, 1921; Opdam y cols., 1976). A partir de los estudios de Woodburne (1939) con técnicas de Marchi, el DCN de los anuros se ha considerado como el sitio de terminación de fibras funiculares dorsales en el rombencéfalo caudal, más que como una entidad citoarquitectónica (Antal y cols., 1980; Nikundiwe y cols., 1982). Nuestros resultados muestran que el DCN se localiza en la placa alar en la región celular que rodea dorsal y lateralmente al polo caudal del tracto solitario, en niveles de transición entre el rombencéfalo y la médula espinal, segregado escasamente de los núcleos del tracto solitario y del tracto descendente del nervio trigémino, situados medial y lateralmente al DCN respectivamente. Sin embargo, una escotadura dorsal sugiere la subdivisión del DCN en un componente medial (*gracilis*) y otro lateral (*cuneatus*) de acuerdo con observaciones previas (Nikundiwe y cols., 1982; Nikundiwe y Nieuwenhuys, 1983).

Quimioarquitectura

En el presente trabajo se han caracterizado diferentes poblaciones celulares en el núcleo de la columna dorsal y en los núcleos adyacentes, mediante la utilización de marcajes histoquímicos e inmunohistoquímicos, en algunos casos combinados con técnicas de trazado neuronal, y se ha realizado la comparación de nuestros resultados con los datos existentes en otros vertebrados.

En mamíferos la sintasa del óxido nítrico (NOS), el cual probablemente juega un papel importante como mensajero neuronal (Bredt y Snyder, 1992; Meller y Gebhart, 1993; Schuman y Madison, 1994), marca una población de neuronas en el DCN que podrían establecer circuitos locales dentro de sus límites (Valtschanoff y cols., 1993). La distribución de la NOS y de la diaforasa neuronal del dinucleótido-fosfato de

nicotinamida y adenina (NADPDd) son idénticas (Bredt y Snyder, 1992), por lo que la NADPHd puede usarse como un marcador para NOS. Nuestros resultados en las especies de anuros estudiadas, demuestran la presencia de neuronas positivas para NADPHd en el DCN, núcleo del tracto solitario y en el núcleo del tracto descendente del nervio trigémino, coincidiendo con datos obtenidos en mamíferos (Leight y cols., el 1990; Vincent y Kimura, 1992; Dohrn y cols., 1994; Takemura y cols., 1994). Igualmente en nuestro materal hemos observado fibras positivas para NADPHd en los tractos descendente del trigémino y solitario, así como en los funículos dorsal y dorsolateral.

Las proteínas ligantes de calcio, como la calbindina D28k (Calb) y la parvoalbúmina (Parv), se expresan en determinadas subpoblaciones neuronales en el sistema nervioso central y periférico (Baimbridge y cols., 1982; Garcia-Segura y cols., 1984; Braun, 1990; Celio, 1990; Ren y Ruda, 1994), e incluso marcan vías enteras y sistemas funcionales completos (Celio, 1990; Andressen y cols., 1993). Estudios recientes (Celio, 1990; Rausell y Jones, 1991a,b; Rausell y cols., 1992; Menétrey y cols., 1992a,b; Maslany y cols., 1992; Ren y Ruda, 1994), han demostrado una distribución preferencial de proteínas ligantes de calcio, como la Calb y la Parv en estructuras somatosensoriales.

En la rata existen neuronas positivas para Calb en determinadas láminas del asta dorsal espinal (Antal y cols., 1990; Ren y Ruda, 1994), incluyendo las células de origen de las proyecciones ascendentes espinales (Menétrey y cols., 1992b), en los núcleos sensitivos trigeminales y en menor medida en los núcleos *gracilis* y *cuneatus* (Celio, 1990; Maslany y cols., 1992). En la rata Menétrey y cols. (1992a) demostraron que las neuronas positivas para Calb constituyen una parte importante de los sistemas de proyección trigeminales y del núcleo del tracto solitario, y Celio (1990) sugirió que la Calb se expresa en toda la vía gustativa. En el mono la Calb se expresa en las proyecciones nociceptivas ascendentes trigeminotalámicas (Rausell y Jones, 1991a,b) y espinotalámicas (Rausell y cols., 1992). En nuestros experimentos, en ambas especies de anuros, se observaron en la región del óbex dos poblaciones neuronales positivas para Calb. La primera en el núcleo del tracto solitario, y la segunda en el núcleo del tracto descendente del nervio trigémino en relación con afarencias primarias trigeminales. En la médula espinal se observaron neuronas positivas para Calb en el asta dorsal. Sin embargo, apenas se observaron neuronas positivas para Calb en el DCN.

Dicho patrón de distribución de neuronas positivas para Calb sugiere que en anfibios anuros, como en mamíferos, la Calb podría estar restringida a la parte nociceptiva del sistema somatosensorial, incluyendo neuronas en el asta dorsal de la médula espinal, y en el núcleo del tracto descendente del trigémino.

La Parv en mamíferos se expresa abundantemente en la vía de la sensibilidad no nociceptiva, es decir, en el sistema columna dorsal-lemnisco medial (Celio, 1990; Rausell y cols., 1992) y en el componente no nociceptivo de las proyecciones trigeminotalámicas (Rausell y Jones, 1991a,b). En nuestros experimentos en anuros, se observó la presencia de una población neuronal diferenciada de células positivas para Parv, que se relacionan con las aferencias primarias espinales y que se asemeja a la población de neuronas de proyección talámica (ver más adelante).

Asimismo, el DCN de los anuros se caracteriza por su contenido en neuronas GABAérgicas, coincidiendo con los datos publicados en mamíferos que describen la presencia de interneuronas GABAérgicas dentro del los núcleos de la columna dorsal (Mugnaini y Oertel, 1985; Rustioni y Weinberg, 1989). Sin embargo, Pritz y Stritzel (1989a) sugirieron que el DCN del reptil (*Caiman crocodilus*) no posee neuronas inmunoreactivas para la descarboxilasa del ácido glutámico (GAD), e indicaron que el DCN en reptiles, al igual que el tálamo dorsal (ver Pritz y Stritzel, 1988), carece de neuronas para la elaboración de circuitos locales.

Igualmente, en el presente estudio se han encontrado algunas neuronas glicinérgicas en el borde ventrolateral del DCN de *Rana perezi*, en línea con datos obtenidos en la lamprea, en la que se han descrito neuronas glicinérgicas que inhiben a neuronas reticuloespinales (Dubuc y cols., 1993a,b), y en mamíferos en los que se han observado células glicinérgicas de distintos tamaños en los núcleos *gracilis* y *cuneatus* (Porucho y cols., 1992).

En experimentos inmunohistoquímicos hemos podido comprobar que la parte más lateral del DCN en anuros, está inervada por fibras inmunoreactivas para sustancia P y CGRP, que ascienden a través del tracto de Lissauer, al igual que en trabajos previos (Rosenthal y Cruce, 1985; Adli y cols., 1988; Petkó y Sánta, 1992). Además de esta proyección peptidérgica, el DCN en anuros está inervado por fibras inmunoreactivas para Leu-encefalina, neuropéptido Y y serotonina, de acuerdo con datos publicados por Ueda y cols. (1984), Merchenthaler y cols. (1989), y Lázár y cols. (1990) en anuros, así como con los resultados obtenidos en mamíferos (Steinbusch, 1981; Westman y cols., 1984; Halliday y cols., 1988; Ibuki y cols., 1989; Tamatani y cols., 1989; Conti y cols, 1990; Fabri y Conti, 1990; Blomqvist y Broman, 1993). Debido a que en nuestros experimentos con aplicaciones de trazadores en el DCN (ver apartado de conectividad) se observaron neuronas retrógradamente marcadas en el núcleo del rafe, rico en neuronas serotoninérgicas (Ueda y cols., 1984), parece probable que este núcleo sea la fuente de la inervación serotoninérgica del DCN, como es el caso en mamíferos (Willcockson y cols., 1987; Blomqvist y Broman, 1993)

Conectividad

Los artículos primero y segundo del presente capítulo se centran en el estudio del sistema columna dorsal-lemnisco medial, y de las proyecciones extralemniscales del núcleo de la columna dorsal (DCN), mediante técnicas de trazado neuronal tanto anterógrado como retrógrado, y demuestran su similitud con el mismo sistema presente en vertebrados amniotas.

Nuestros resultados en anuros corroboran, de acuerdo con estudios previos (Antal y cols., 1980; Nikundiwe y cols., 1982; Jhaveri y Frank, 1983), la existencia un sistema de aferencias primarias espinales, somatotópicamente organizado, que alcanza y delimita el DCN, de forma que la región medial (*gracilis*) está inervada por fibras procedentes de segmentos corporales lumbares y torácicos, mientras que las fibras de segmentos cervicales proyectan a la región lateral (*cuneatus*). En el caso de las aferencias braquiales las fibras primarias continúan rostralmente para inervar el complejo nuclear vestibular y, en mayor número, la capa granular del cerebelo (Antal y cols., 1980; Székely y cols., 1980).

La presencia en el funículo dorsal de aferencias espinales no primarias a los núcleos de la columna dorsal, o sistema postsináptico de la columna dorsal (PDCS), se ha demostrado en mamíferos (Rustioni, 1973; Angaut-Petit, 1975a,b; Rustioni y Kaufman, 1977; Bennett y cols., 1984; Giesler y cols., 1984), aves (Funke, 1988) y reptiles (Pritz y Stritzel, 1994). En algunos mamíferos el PDCS transmite información nociceptiva (Uddenberg, 1968; Angaut-Petit, 1975b; Bennett y cols., 1984; Kamogawa y Bennett, 1986). En anbibios ten Donkelaar y de Boer-van Huizen, (1991) sugirieron su existencia por primera vez en un estudio realizado en larvas de *Xenopus laevis*. Nuestros resultados en experimentos con aplicaciones de trazadores al DCN confirman, en adultos tanto de *Xenopus laevis* como *Rana perezi*, la presencia de aferencias espinales no primarias al DCN, las cuales se originan mayoritariamente en la sustancia gris dorsal ipsilateral, en toda la extensión rostrocaudal de la médula espinal, y se organizan de manera somatotópica, como en el caso de las aferencias primarias.

Igualmente hemos observado que la parte lateral del DCN de los anuros está inervada por fibras del tracto descendente del nervio trigémino, procedentes de los nervios trigémino, facial, glosofaringeo y vago, de acuerdo con resultados obtenidos en estudios previos (Rubinson y Friedman, 1977; Matesz y Székely, 1978; Fuller, 1979; Lowe y Russell, 1982; Altman y Dawes, 1983; Stuesse y cols., 1984; Oka y cols., 1987; González y cols., 1993).

En mamíferos la transmisión de información sensitiva, mediante el sistema columna dorsal-lemnisco medial, es controlada por vías procedentes de la corteza cerebral (Kuypers, 1958; Kuypers y Tuerck, 1964), núcleo rojo (Edwards, 1972; Weinberg y Rustioni, 1989), núcleos vestibulares (Weinberg y Rustioni, 1989), cerebelo (Sotgíu y Cesa-Bianchi, 1972), y formación reticular (Willcockson y cols., 1987; Weinberg y Rustioni, 1989). Nuestros resultados demuestran que en anuros, con la excepción de la corteza cerebral y del núcleo rojo, existe un "control" del DCN comparable al de mamíferos. En experimentos con inyecciones de trazadores en el DCN, se observó un marcaje bilateral en células del núcleo cerebeloso, núcleo ventral del nervio VIII y en la formación reticular en niveles entre los núcleos motores de los nervios VII y IX, así como en el núcleo inferior del rafe cuya proyección, presumiblemente serotonérgica, podría ser la fuente de la fuerte inervación inmunoreactiva para serotonina del DCN, de acuerdo con los datos descritos en mamíferos (Willcockson y cols., 1987; Blomqvist y Broman, 1993).

Una parte del presente estudio se ha centrado en las conexiones eferentes del DCN. Aunque son escasos en la literatura los trabajos sobre el lemnisco medial en anuros, diversos autores presentaron evidencias anatómicas y electrofisiológicas de la existencia una proyección contralateral desde la región del DCN al tálamo (Vesselkin y cols., 1971; Vesselkin y Kovacevic, 1973; Silvey y cols., 1974; Neary y Wilczynski, 1977; Urbán y Székely, 1982), y a la región lateral del torus semicircularis (Comer y Grobstein, 1981; Wilczynski, 1981; Neary y Wilczynski, 1986; Neary, 1988), lo que sugiere que el lemnisco medial, se asemeja al presente en amniotas, si bien no existen, hasta el momento, estudios basados en técnicas de trazado anterógrado sobre la anatomía detallada del lemnisco medial, que lo confirmen.

En nuestros experimentos, con aplicaciones de trazadores en el DCN, se observó el lemnisco medial como un sistema mayoritariamente contralateral que asciende a lo largo del tronco cerebral hasta el

diencéfalo. En su recorrido origina colaterales que alcanzan diversas zonas de la formación reticular rombencefálica, el área octavolateral, y la capa granular del cerebelo. En niveles mesencefálicos, el lemnisco medial inerva la región lateral del torus semicircularis, principalmente los núcleos laminar y magnocelular, y los núcleos tegmentales anterodorsal y anteroventral, así como los núcleos rojo e intersticial del fascículo longitudinal medial. En *Xenopus laevis*, hemos observado que las capas tectales intermedias y profundas están también inervadas, de acuerdo con datos previos de trazado retrógrado (Wilczynski y Northcutt, 1977; Zittlau y cols., 1988; Hofmann y cols., 1990; Masino y Grobstein, 1990). En niveles rostrales mesencefálicos, algunas fibras marcadas se distribuyen en la sustancia gris pretoral y gris pretectal. En el diencéfalo, diversas áreas talámicas, tanto dorsales como ventrales, reciben fibras del lemnisco medial. La parte ventral de los núcleos posterior, central y, en menor medida, del núcleo anterior del tálamo dorsal están inervadas, mientras que los núcleos ventromedial y ventrolateral talámicos así como el núcleo del tubérculo posterior reciben una inervación más densa.

Debido a la escasa diferenciación de los distintos componentes presentes en la placa alar de la región del óbex, y con el fin de confirmar si las citadas proyecciones ascendentes realmente se originan en el DCN, se realizaron aplicaciones de diversos trazadores en el tálamo ventral, torus semicircularis y en el cerebelo en ambas especies de anuros.

En los experimentos con aplicaciones de trazadores en el tálamo se observaron células marcadas retrógradamente en la región del DCN, mayoritariamente en el lado contralateral. Las dendritas de dichas neuronas son largas y se dirigen tanto dorsal como ventrolateralmente, alcanzando los funículos dorsal y dorsolateral, respectivamente. Sus axones se pueden seguir en el lemnisco medial contralateral. Las aplicaciones realizadas en el torus semicircularis permitieron marcar neuronas dentro del DCN, principalmente contralaterales. Se observaron dos grupos celulares distintos cuya segregación es más patente en *Rana perezi* que en *Xenopus laevis*. El primero está constituído por células localizadas en la región más dorsal de la sustancia gris, con dendritas que se extienden en la zona de fibras situada dorsalmente a ellas. Sus axones se dirigen ventromedialmente, para cruzar la línea media y formar parte del lemnisco medial. El segundo grupo de células marcadas se localiza en la zona lateral marginal de la sustancia gris dorsal, desde el nivel del óbex al segundo segmento espinal, sus dendritas se dirigen principalmente a la parte dorsal del funículo lateral y al funículo dorsal, y sus axones ingresan en el lemnisco medial.

Además, en los experimentos con aplicaciones tanto en el tálamo como en el torus semicircularis, se marcaron bilateralmente algunas células en el núcleo del tracto descendente del nervio trigémino, coincidiendo con datos previos (Comer y Grobstein, 1991; M.Muñoz y cols., 1994).

El componente ventrolateral del DCN proyecta al tálamo y al torus semicircularis y se extiende caudalmente hasta el segundo segmento espinal. Las dendritas de sus neuronas se dirigen principalmente al funículo dorsolateral, mientras que sus axones se incorporan al lemnisco medial contralateral. Se podría establecer una comparación con el núcleo cervical lateral de los mamíferos, el cual recibe información somatosensorial a través del tracto espinocervical y proyecta, contralateralmente a través del lemnisco medial, a regiones somatosensoriales mesencefálicas y talámicas (Willis y Coggeshall, 1991).

El presente estudio confirma la presencia en anuros del sistema columna dorsal-lemnisco medial cuyas dianas rombencefálicas, mesencefálicas y diencefálicas son mucho más diversas y extensas de lo que se había sugerido en estudios previos (Vesselkin y cols., 1971; Silvey y cols., 1974; Neary y Wilczynski, 1977; Comer y Grobstein, 1981; Wilczynski, 1981; Forehand y Farel, 1982; Urbán y Székely, 1982; Neary, 1988). La vía lemniscal en anuros parece ser, en líneas generales, similar a la de amniotas (reptiles: Ebbesson, 1978; Siemen y Künzle, 1994a; aves: Wild, 1989; mamíferos: Hazlett y cols., 1972; Hand y van-Winkle, 1977; Feldman y Kruger, 1980; Berkley y cols., 1986; ver también Willis y Coggeshall, 1991)

Además del lemnisco medial, en nuestro material con aplicaciones de trazador en el DCN, observamos que éste núcleo origina proyecciones extralemniscales ipsilaterales a la corteza cerebelosa, y proyecciones bilaterales a la médula espinal. En experimentos de trazado retrógrado, con aplicaciones en el cerebelo, observamos neuronas marcadas bilateralmente en el DCN, aunque en mayor número en el lado ipsilateral de acuerdo con datos previos (González y cols., 1984). Los axones de dichas neuronas parecen discurrir junto con las fibras primarias espinales que ascienden igualmente hasta la capa granular del cerebelo. Las neuronas del DCN que proyectan a la médula espinal envían sus axones a través del funículo dorsal ipsilateral, y terminan en el asta dorsal y de forma más dispersa en los campos lateral y ventral, principalmente en niveles cervicales. Dicha proyección fue igualmente confirmada en experimentos de trazado retógrado, con aplicaciones en diversos niveles espinales, en los que se marcó una población celular en el DCN ipsilateral.

En el tercer artículo de este capítulo se presentan evidencias en favor de la existencia en anuros del sistema espino-cervico-talámico, descrito en mamíferos y formado por el tracto espinocervical que termina en el núcleo cervical lateral (LCN), y los tractos cervicomesencefálico y cervicotalámico respectivamente que forman parte del lemnisco medial (ver Willis y Coggeshall, 1991). Hasta el momento, el sistema espino-cervico-talámico no ha sido descrito en vertebrados no mamíferos, en los que únicamente se dispone de datos aislados que sugieren su existencia (Ebbesson, 1967; Finger, 1981; Forehand y Farel, 1982; Ito y cols., 1986; Necker, 1989; Ronan y Northcutt, 1990).

En anfibios el tracto espinocervical no ha sido descrito, aunque en estudios basados en técnicas degenerativas se observó la presencia de proyecciones espinales ascendentes en el funículo dorsolateral (Ebbesson, 1976). Algunas de dichas proyecciones corresponden a aferencias primarias del tracto de Lissauer (Antal y cols., 1980; Nikundiwe y cols., 1982), si bien podrían igualmente incluir fibras no primarias del tracto espinocervical.

En el presente estudio se han realizado experimentos *in vitro* de trazado anterógrado y retrógrado en *Xenopus laevis*, con objeto de caracterizar el patrón de terminación del posible tracto espinocervical, así como las células que lo originan.

En experimentos con aplicaciones de BDA, en la región dorsal de la médula espinal en segmentos lumbares, torácicos y cervicales, se observaron tractos de fibras en los funículos dorsal y dorsolateral, rostral y caudalmente a los sitios de inyección.

Las fibras que ascienden en el funículo dorsal presentan un ordenamiento somatotópico, de acuerdo con los datos descritos para las proyecciones primarias (Antal y cols., 1980; Nikundiwe y cols., 1982; Jhaveri y Frank, 1983) y no primarias (A.Muñoz y cols., 1995), y emiten colaterales que alcanzan principalmente los campos dorsal y lateral y ocasionalmente los campos ventrales en distintos segmentos espinales, dependiendo del nivel de la aplicación. En la región del óbex dichas fibras inervan el DCN ipsilateral, de acuerdo con la somatotopía mediolateral que presentan en el funículo dorsal.

Las fibras del funículo dorsolateral originan, rostral y caudalmente al nivel de inyección, colaterales que inervan los campos dorsal y lateral de distintos segmentos espinales, según el nivel de la aplicación. Algunas de estas fibras podrían ser aferencias primarias espinales pertenecientes al tracto de Lissauer (Antal y cols., 1980; Nikundiwe y cols, 1982), aunque también podrían existir en anfibios proyecciones espinales no primarias intersegmentarias a través del funículo dorsolateral, como ocurre en mamíferos, en los que las neuronas del tracto espinocervical emiten colaterales a diversas dianas en distintos niveles espinales (Snow y cols., 1976; Brown y cols, 1977; Rastad y cols, 1977; Jankowska y cols., 1979; Maxwell y Koerber, 1986; Cao y cols., 1993).

En segmentos cervicales superiores y a nivel del óbex, la mayoría de las fibras del funículo dorsolateral, procedentes de todos los niveles espinales, se tuercen dorsomedialmente para inervar masivamente las neuronas situadas en el límite ventrolateral del asta dorsal. Dicha proyección representa en anfibios un posible equivalente del tracto espinocervical, presente en mamíferos (Willis y Coggeshall, 1991). En niveles ligeramente caudales al óbex se observó una región en la sustancia gris, con forma de banda, en la que se produce un solapamiento de las proyecciones, procedentes de los funículos dorsal y dorsolateral, que inervan el DCN y el equivalente en anfibios del LCN respectivamente (ver más adelante). Dicha región delimita el borde ventral del tracto descendente del trigémino, y de las células relacionadas con él (González y cols., 1993).

El tracto espinocervical en mamíferos es una proyección glutamatérgica (Broman y cols., 1990; Kechagias y Broman, 1994) que se origina en células espinales que reciben proyecciones desde la periferia (Willis y Coggeshall, 1991). Dichas neuronas se distribuyen ipsilateralmente en el núcleo propio, la sustancia gelatinosa de Rolando, láminas IV, V, y en niveles cervicales en las laminas I, VI y VII; y contralateralmente en las láminas I, VII y VIII (Brown y cols, 1980; Baker y Giesler, 1984; Craig, 1976, 1978; Craig y cols., 1992).

En vertebrados no mamíferos el tracto espinocervical no se ha descrito como tal, si bien existen evidencias, basadas en experimentos de degeneración y de trazado anterógrado y retrógrado, en favor de su existencia en aves (van den Akker, 1970; Funke y Necker, 1986; Funke 1988; Necker, 1991) y reptiles (Ebbesson; 1967). En agnatos, (Northcutt y Ebbesson, 1980; Ronan y Northcutt, 1990), así como en peces cartilaginosos (Hayle, 1973a,b; Ebbesson y Hodde, 1981; Smeets y cols., 1984) y óseos (Hayle 1973a,b; Finger, 1981), se ha demostrado la presencia de proyecciones espinales ascendentes a través de la región dorsal del funículo lateral, aunque no se ha descrito el tracto espinocervical.

Con objeto de caracterizar la población neuronal cuyas proyecciones ascienden en el funículo

dorsolateral, se realizaron experimentos *in vitro* con pequeñas aplicaciones de BDA en el funículo dorsolateral, a nivel del óbex y en el segundo segmento espinal cervical. Dichos experimentos marcaron una extensa población de células en el asta dorsal, principalmente ipsilateral, desde niveles cervicales hasta niveles sacros. La mayoría de las células se encuentran en la región más profunda del campo dorsal de la sustancia gris espinal. Igualmente se observan neuronas marcadas, aunque en menor número, ipsilateralmente en la parte superficial del asta dorsal, y contralateralmente en los campos ventrales de la sustancia gris, en segmentos cervicales inferiores y torácicos.

El LCN en mamíferos está formado por neuronas adyacentes así como incluídas dentro del funículo dorsolateral, y se extiende desde el nivel del óbex hasta el segundo segmento espinal. Los axones de sus neuronas ascienden a través del lemnisco medial y alcanzan, principalmente, la región intercolicular mesencefálica somatosensorial, así como el complejo ventrobasal del tálamo, formando los tractos cervicomesencefálico y cervicotalámico, respectivamente (Willis y Coggeshall, 1991).

Aunque en aves no ha sido descrito como tal núcleo, existen evidencias que indican la existencia del LCN (Ramón y Cajal, 1911; Karten y Hodos, 1967; van den Akker, 1970; Necker, 1989, 1990), sin embargo, en reptiles no se ha mencionado nada respecto a la posible existencia del LCN (Ebbesson 1967, 1969; Kusuma, 1979; Künzle y Woodson, 1982; Pritz y Stritzel, 1986; Hoogland, 1981, 1982; Pritz y Stritzel, 1989, el 1990).

En anamniotas los datos sobre la presencia de un LCN son escasos. En teleósteos, se ha descrito un núcleo comparable a nivel del óbex y en los primeros segmentos espinales cervicales (Finger, 1981; Ito y cols., 1986). En *Sebasticus marmoratus* Ito y cols. (1986) describieron el LCN en base a sus proyecciones al núcleo ventromedial talámico. Adicionalmente, en lampreas, en experimentos con aplicaciones diencefálicas o mesencefálicas de HRP, se observaron neuronas marcadas retrógradamente a nivel del óbex y en los primeros segmentos cervicales espinales, sugieriéndose la presencia de un posible LCN en agnatos (Ronan y Northcutt, 1990).

En anuros, mediante estudios basados en tinciones de Nissl (Opdam y cols., 1976; Nikundiwe y Nieuwenhuys, 1983, o de Golgi (Ebbesson, 1976), no se ha descrito el LCN como una entidad citoarquitectónica definida. Sin embargo, la existencia del LCN fue sugerida en un trabajo sobre el desarrollo ontogénico de la médula espinal (Forehand y Farel, 1982), en base al marcaje retrógrado observado en experimentos en los se realizaron aplicaciones de HRP en la región lateral de la formación reticular rombencefálica, y en el mesencéfalo; asimismo A. Muñoz y cols. (1995; artículo segundo del presente capítulo), mediante aplicaciones de trazadores en el tálamo y en el torus semicircularis, marcaron retrógradamente neuronas, separadas ventrolateralmente de las del DCN en *Rana perezi*, y parcialmente entremezcladas con ellas en *Xenopus laevis*, que fueron consideradas entonces como un componente ventrolateral de este núcleo, con cierta similitud al LCN por su relación con el funículo dorsolateral.

En el tercer artículo del presente capítulo se realizaron experimentos adicionales con aplicaciones de BDA en el tálamo ventral o en el torus semicircularis. En ambos tipos de experimentos se marcó retrógradamente una población neuronal en un área de la placa alar de la región del óbex, que incluye dos

componentes no distinguibles citoarquitectónicamente: el DCN, localizado dorsomedialmente y en relación con las fibras ascendentes del funículo dorsal, y el LCN en posiciones más ventrolaterales donde terminan masivamente las fibras del funículo dorsolateral. Aunque solo algunas células del LCN están realmente localizadas dentro del propio funículo dorsolateral, las dendritas de todas ellas se dirigen mayoritariamente en dirección ventrolateral, extendiéndose a lo largo del funículo dorsolateral, a través del cuál pueden recibir información espinal ascendente. No obstante, a nivel del óbex, donde coexisten el DCN y el LCN, es difícil discernir a cuál de las dos poblaciones pertenecen las neuronas localizadas en posiciones intermedias, únicamente la orientación de sus dendritas, dorsalmente hacia el funículo dorsal en el caso de neuronas del DCN, o ventrolateralmente hacia el funículo dorsolateral en el caso de las del LCN, así como la extensión más rostral del primer núcleo, permiten establecer diferencias

En el presente capítulo se presentan evidencias que demuestran la existencia en anuros de los sistemas columna dorsal-lemnisco medial y espino-cervico-talámico, con un patrón básico de organización, similar al descrito en amniotas. Si bien existen datos que sugieren una organización similar en peces, no se han realizado estudios recientes, mediante técnicas de trazado neuronal e inmunohistoquímicas que lo demuestren, por lo que resulta necesario su estudio con objeto de establecer si existe un esquema similar en la anatomía de sistemas somatosensoriales en todos los vertebrados.

CAPÍTULO 6

Organization of the caudal rhombencephalic alar plate of the ribbed newt Pleurodeles waltl: Evidence for the presence of dorsal column and lateral cervical nuclei

6.1

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ABSTRACT

As part of a research programme on the evolution of somatosensory systems in vertebrates, the cytoarchitecture, chemoarchitecture, and fiber connections of the caudal rhombencephalic alar plate were studied in the ribbed newt, *Pleurodeles waltl*. This part of the brain stem includes ill-defined dorsal column and lateral cervical nuclei. A cytoarchitectonic analysis revealed that the caudal medullary alar plate consists of an inner and an outer cell layer. The outer cell layer at the obex level forms the dorsal column nucleus (DCN), whereas the ventrolateral part of this cell layer forms a lateral cervical nucleus (LCN). NADPH-diaphorase histochemistry and calbindin D-28k immunohistochemistry clearly delineate the main components of the compact inner cell layer, i.e. the nucleus of the solitary tract dorsally and the nucleus of the descending trigeminal tract ventrally. Neither NADPH-diaphorase-labeled nor calbindin D-28k

positive neurons were observed in DCN and LCN. With anterograde and retrograde tracing the DCN and LCN were further delineated. Labeling of ascending dorsal root afferents showed that the dorsal column and the DCN are somatotopically arranged: lumbar primary afferents terminate on medial DCN neurons, whereas cervical primary afferents terminate on lateral DCN neurons. The LCN is densely innervated by the dorsolateral funiculus. Retrograde tracing showed extensive predominantly contralateral projections of both the DCN and LCN to the torus semicircularis and the ventral thalamus.

These data show that even in the poorly segregated caudal rhombencephalic alar plate of urodeles a DCN and LCN can be distinguished with afferent and efferent projections comparable to those in anurans and other terrestrial vertebrates.

INTRODUCTION

Ascending spinal projections in the brain of vertebrates include three main systems: 1) mainly ipsilateral primary and non-primary (i.e. the postsynaptic dorsal column system) projections via the dorsal funiculus that terminate primarily in the dorsal column nucleus within the alar plate of the most caudal part of the medulla; 2) ipsilateral, non-primary projections via the dorsolateral funiculus that terminate at upper cervical segments, in the lateral cervical nucleus and in various rhombencephalic and mesencephalic targets; 3) secondary projections via the anterolateral column of ventral quadrant, i.e. the ventral and ventrolateral funiculi, to the reticular formation, mesencephalon and thalamus (A. Muñoz et al., 1995, 1996a,b). Additionally, both the dorsal column and the lateral cervical nuclei give rise to contralateral and, to a lesser extent, ipsilateral, projections to the mesencephalon and thalamus through the medial lemniscus (Willis and Coggeshall, 1991).

In urodeles, in previous studies based on silver staining (Herrick, 1914, 1930) and on anterograde degeneration (Nieuwenhuys and Cornelisz, 1971) techniques, spinal ascending fibers in the ventral and lateral funiculi were described that reach rhombencephalic and mesencephalic targets. Recent studies based on modern tract-tracing techniques demonstrated a more elaborate pattern of spinal fibers that ascend in the ventral quadrant of the spinal cord (A. Muñoz et al., 1994a). In anurans, the dorsal column nucleus (DCN) is characterized by its afferent (Antal et al., 1980; Nikundiwe et al., 1982; ten Donkelaar and de Boer van Huizen, 1991; A. Muñoz et al., 1995) and efferent (Neary and Wilczynski, 1977; Forehand and Farel, 1982; A. Muñoz et al., 1994b, 1995) connections, as well as immunohistochemically (A. Muñoz et al., 1995) and electrophysiologically (Silvey et al., 1974; Urbán and Székely, 1982). Moreover, in anurans, the presence of a spinocervical tract and other ascending spinal projections in the dorsolateral funiculus, as well as the existence of a lateral cervical nucleus (LCN) and its mesencephalic and diencephalic medial lemniscal projections were described recently (A. Muñoz et al., 1996a). In urodeles, studies based on silver staining (Herrick, 1914, 1930, 1944), anterograde degeneration (Nieuwenhuys and Cornelisz, 1971) and tract-tracing (Roth and Wake, 1985) techniques suggest that spinal primary afferent projections to the dorsal gray at the obex level are somatotopically organized. In the tiger salamander, *Ambystoma tigrinum*, Herrick (1944) reported the presence of a poorly segregated nucleus commissuralis in the obex region related to visceral afferents of the solitary tract, and a nucleus of the funiculus dorsalis probably receiving lateral line,

vestibular, trigeminal and, especially, ascending spinal afferents. Neither a DCN nor an LCN were noted in a later cytoarchitectonic study of the brain stem in the axolotl (Opdam and Nieuwenhuys, 1976).

The central nervous system of urodeles is often considered to be primitive. However, careful studies by e.g. Roth and co-workers (summarized by Roth, 1987 and Roth et al., 1993) show that the salamander brain possesses virtually all the anatomical and functional properties found in anurans, which are usually regarded as being much more evolved with respect to the guidance of comparable behavior. Recent studies (e.g., Northcutt, 1987; Roth et al., 1992, 1993) suggest that the evolution of the salamander nervous system is characterized by *secondary simplification*, which gives the impression that the brains of salamanders are more primitive than the phylogenetic position of salamanders, as tetrapods, implies (Roth et al., 1992). In the present study, an attempt is made to characterize the various neuronal components of the caudal part of the medullary alar plate in the ribbed newt, *Pleurodeles waltl*. It can be expected that within this ill-defined part of the brain a DCN and an LCN with appropriate fiber connections are present. The present study includes: 1) a discussion of the cytoarchitectonics of the caudal part of the medullary alar plate; 2) data on the chemical neuroanatomy of this ill-defined area, and 3) an analysis of its fiber connections.

MATERIALS AND METHODS

The data presented are based on a total of 77 adult speciments of *Pleurodeles waltl* obtained from laboratory stock of the Department of Cell Biology, University Complutense of Madrid or donated by Dr. Gerhard Roth from the University of Bremen (Germany). For a cytoarchitectonic analysis of the obex region, Nissl (cresylecht violet)-stained series were available, cut either transversally, horizontally or sagittally at a thickness of 20 µm. The histochemical, immunohistochemical and tract-tracing techniques used in this study are discussed below. The nomenclature used is based on studies by Opdam and Nieuwenhuys (1976) on the brain stem.

NADPH-diaphorase histochemistry

Eight animals were anesthetized in a 0.3% solution of tricaine methanesulphonate (MS222, Sandoz), and subsequently perfused transcardially with a 0.9% saline solution followed by a fixative containing 4% paraformaldehyde and 15% saturated picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were taken out and further fixed in the same fixative for six to eight hours at room temperature. They were subsequently immersed in a 30% phosphate buffer solution at 4°C, embedded in a 15% gelatin and 30% sucrose solution, and stored for five hours in a 4% formaldehyde solution at room temperature. On a freezing microtome, 30 or 40 µm frontal sections were cut and collected in phosphate buffer. Free-floating sections were incubated in a medium containing 1mM ß-NADPH, 0.8mM nitroblue tetrazolium and 0.06% Triton X-100 in 0.1M phosphate buffer (pH 7.6) at 37°C for one to two hours. After incubation, the sections were thoroughly rinsed in PB. In three cases after rinsing, the sections were also processed for tyrosine hydroxylase immunohistochemistry as described below.

Immunohistochemical procedures

For the immunohistochemical procedures used, animals were anesthetized with an overdose of MS222, and transcardially perfused with saline followed by a mixture of 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1M phosphate buffer (pH 7.4). The brain and spinal cord were removed, postfixed for 4-7 hours in the same fixative and embedded in 15% gelatin with 30% sucrose. Brains were cut frontally on a freezing microtome or on a vibratome at 40 µm, and the sections were collected in Tris-saline (TBS) buffer (0.05M, pH 7.6). All antibodies were diluted in 0.1% normal serum of the species in which the secondary antibody was raised in TBS with 0.1% Triton X-100 (Sigma). The sections were preincubated for 1-2h in TBS containing 3% normal serum and 0.1% Triton X-100 and subsequently incubated in the primary antibody-containing solution for 12-60h at 4°C. Controls for the immunohistochemistry experiments included: 1) staining some selected sections with pre-immune mouse serum (1:1,000 for tyrosine hydroxylase, and calbindin D-28k immunohistochemistry), or with rabbit serum (1:1,000 for neuropeptide Y and serotonin; 1:2,000 for substance P and Leu-enkephalin immunohistochemistry) instead of the primary antibody, and 2) controls in which either the primary antibody, secondary antibody or the peroxidase-antiperoxidase complex (PAP) was omitted. As an additional control for the specificity of the labeling of calcium-binding proteins, some sections were stained using antibodies that had been pre-absorbed in an excess of calbindin (Sigma). The sections were processed according to the peroxidase-antiperoxidase (PAP) technique (Sternberger, 1979) in a series of incubations with the following antisera:

1) *tyrosine hydroxylase (TH) immunohistochemistry (8 cases):* a) mouse anti-TH (Incstar), diluted 1:1,000, for 24-72 hours; b) goat anti-mouse (Nordic), diluted 1:100, for three to five hours, and c) rat peroxidase-antiperoxidase (PAP) complex (Incstar), diluted 1:500 for two hours.

2) *Nitric oxide synthase (NOS): (5 cases)* a) sheep anti-NOS (gift from Dr. Emson), diluted 1:20,000, for 36-60 hours, b) biotinylated rabbit anti-sheep diluted 1:200 for two hours at room temperatute and c) ABC Elite Kit (Vector) for 1.5h at room temperature. For fluorescent immunolabeling a rhodamine-coupled donkey anti-sheep (Chemicon) or a fluorescein-coupled rabbit anti-sheep (Vector) were used as second antibody diluted 1:100 for two hours.

For double TH/NOS immunofluorescence labeling (3 cases) the section were simultaneusly incubated in a solution containing the same first antibodies as for single labeling (TH and NOS). After rinsing, sections were incubated first in a solution containing biotynilated horse anti-mouse (Vector) diluted 1:100 for one hour at room temperature, and then, after rinsing in a mixture containing a Texas red-coupled streptavidin complex (Vector) diluted 1:100 and a rabbit anti-sheep Fluoresceine-coupled (Vector) diluted 1:75 for two hours at room temperature.

3) *calbindin D-28k immunohistochemistry (5 cases):* a mouse anti-calbindin D-28k (Sigma), diluted 1:1,000 for 24-72 hours; b) goat antimouse (Nordic), diluted 1:100, for three to five hours, and c) rat PAP complex (Incstar), diluted 1:500, for two hours.

4) *Substance P (SP) (5 cases) and Leu-enkephalin (L-Enk) immunohistochemistry (4 cases):* a) rabbit anti-SP or rabbit anti-L-Enk (CRB), diluted 1:2,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for two hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for two hours;

5) *Neuropeptide Y (NPY) immunohistochemistry (4 cases):* a) rabbit anti-NPY serum (gift from Dr. J.D. Mikkelsen), diluted 1:1,000, for 36 hours; b) swine anti-rabbit (Nordic), diluted 1:50, for one hour, and c) rabbit PAP complex (Dakopatts), diluted 1:800, for one hour;

6) *serotonin (5-HT) immunohistochemistry (5 cases):* a) rabbit anti 5-HT (gift from Dr. H.W.M. Steinbusch), diluted 1:1,000, overnight; b) swine anti-rabbit (Nordic), diluted 1:50, for two hours, and c) rabbit PAP complex (Dakopatts), diluted 1:600, for two hours.

In all cases, after rinsing, the sections were incubated with 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 0.01% H_2O_2 in phosphate buffer, for 10-15 minutes. After another rinsing, the sections were mounted on glass slides, dried overnight, and coverslipped. In most cases, visualization of the immunostaining was improved by processing the sections with nickel-enhanced DAB (0.05% DAB, 0.01% H_2O_2, 0.04% ammonium nickel sulphate in phosphate buffer).

Tract tracing experiments

For all the surgical procedures the animals were anesthetized by inmersion in a 0.3% solution of tricaine methanesulphonate (MS222, Sandoz) in tap water. For the tract-tracing experiments the bidirectionally transported tracer biotinylated dextran amine (BDA 10kD, Molecular Probes) was either iontophoretically (5-8 μA, 7 sec. on/7 sec. off) injected (a 10% solution in 0,1 phosphate buffer, pH 7.4) through a glass micropipette (outer tip 20-30 μm) or applied recrystallized at the tip of fine tungsten needles or glass micropipettes, to the dorsal part of the cervical spinal cord (7 cases), to the ventral thalamus (5 cases) and to the torus semicircularis (5 cases). The animals were allowed to survive for 7-10 days. They were then reanesthetized with an overdose of MS222 and perfused transcardially with saline followed by 200ml of 4% paraformaldehyde in PB. In all cases the brain and spinal cord were removed and further fixed for 3-7h, cryoprotected in 30% sucrose in PB for 3-5h at 4°C and embedded in a medium of 15% gelatin with 30% sucrose in PB. The blocks were fixed for 7h in a 2% formaldehyde, 30% sucrose. Sections were cut transversally at 40 μm on a freezing microtome and collected in PB. For visualizing BDA, an avidine biotin complex (Vectastain ABC Standard Kit, Vector Laboratories) was used. Histochemistry for HRP followed the heavy metal intensification of the diaminobenzidine (DAB)-based HRP reaction product according to Adams (1981).

Selected sections from tract-tracing, histochemical and immunohistochemical experiments were counterstained with 1% cresyl violet or toluidine blue in distilled water. After rinsing all the sections were mounted on glass slides (mounting medium 0.25% gelatin in Tris buffer), dried, and coverslipped with Entellan (Sigma) or with Vectashiel (Vector) for experiments using fluorescence microscopy .

RESULTS

Delineation and (immuno)histochemical characterization of cell groups in the urodele caudal rhombencephalic alar plate.

Cytoarchitecture

In the alar plate of the caudal medulla of *Pleurodeles waltl*, at obex levels, evidence for different

cell components such as a dorsal column nucleus (DCN), a lateral cervical nucleus (LCN), a nucleus of the solitary tract and a nucleus of the descending tract of the trigeminal nerve can be observed in Nissl-stained material (Fig. 1).

The neurons in the alar plate in *Pleurodeles waltl* form a curved, continuous zone of periventricular gray in which individual cell groups are difficult to recognize. However, in the caudal brain stem and upper spinal cord levels a distinct cellular band (outer cell layer), oriented dorsomedially-to-ventrolaterally, separated from the main periventricular layer (inner cell layer) by a thin layer of white substance, can be distinuished (Figure 1B,C). In the upper cervical cord just caudal to the obex, the inner cell layer is rather thick and compact, whereas in its outer part a thinner cell layer (outer layer) is present although a clear boundary between the two layers can not be observed (Figure 1D,E). The outer cell layer is more evident dorsomedially where the cells form what will be tentatively considered as the DCN since they are closely related to the site of termination of the ascending dorsal funicular spinal fibers (Figure 1D,E). At the ventrolateral aspect of the outer cell layer, neurons are more sparsely organized and related to the fibers passing via the dorsolateral funiculus. Some of them are located even within the dorsolateral funiculus. This ventrolateral cluster of neurons forms what will be termed the LCN (Figure 1B,C). Here, a thin lamina of white matter separates the inner and the outer cell layers. Rostral to the obex, this separation is more evident and due to the presence of the solitary tract (Figure 1A). The DCN is found dorsal to the solitary tract and extends as far rostrally as the motor nucleus of the Xth nerve, where its cells become intermingled with cells of the caudal part of the lateral line area. The DCN consists of small (8-10 μm) and medium-sized (15 μm) multipolar neurons. No medial ('gracile') and lateral ('cuneate') compartments can be distinguished.

The ventrolateral aspect of the DCN extends into areas where its cells are difficult to distinguish from those of the LCN or even the nucleus of the descending trigeminal tract. The latter nucleus and the nucleus of the solitary tract are located within the dense inner cell layer. They are rather poorly segregated from each other or from the reticular elements located ventromedially. In general, neurons of the nucleus of the solitary tract are located dorsomedial to the trigeminal neurons. The nucleus of the descending trigeminal nerve can be characterized by the presence of trigeminal afferents in tract-tracing experiments.

Chemoarchitecture

NADPHd histochemistry

In the caudal part of the rhombencephalic alar plate of *Pleurodeles waltl*, just rostral to the obex, NADPHd-positive neurons were observed in the outer half of the inner cell layer (Fig. 2). This cell population forms part of the nucleus of the descending trigeminal tract and extends caudally up to the cervical spinal cord. It contains medium-sized, round-to-oval neurons with long NADPHd-positive processes directed dorsolaterally towards the descending tract of the trigeminal nerve. Additionally, NADPH-positive cells were found in the nucleus of the solitary tract intermingled with catecholaminergic neurons, in experiments in which NADPHd-staining was combined with immunohistochemistry against tyrosine hydroxylase. However, NADPHd-positive neurons in the nucleus of the solitary tract were more numerous rostrally. At the obex level only a few positive cells were observed in the outer half of the inner cell layer.

No NADPH-positive cells were found in the area of the DCN. NADPHd-positive fibers were found predominantly in two bundles, i.e. the descending trigeminal tract and the rostral portion of the solitary tract. Weakly labeled fibers were also present in the dorsal and dorsolateral funiculi close to the obex region. Heavier staining within these funiculi is present in the spinal cord.

Immunohistochemical data

The distribution of nitric oxide synthase (NOS)-immunopositive neurons in the caudal part of the rhombencephalic alar plate is shown in Figure 3, the presence of tyrosine hydroxylase-positive neurons in Figure 4, and the distribution of the calcium-binding protein calbindin D-28k in Figure 5.

Nitric Oxide Synthase (NOS): The distribution of NOS-immunopositive neurons in the medullary alar plate of *Pleurodeles waltl* is comparable to that observed after NADPHd-staining. Weak labeling was observed in the nucleus of the descending trigeminal tract. Neurons with long processes directed dorsolaterally towards the descending trigeminal tract were observed. Additionally, some, more dorsomedially located, neurons were observed that may belong to either the nucleus of the solitary tract or the DCN.

Tyrosine hydroxylase (TH): TH immunohistochemistry (Figure 4) revealed, in line with previous studies (González and Smeets, 1991, 1995), the presence of catecholaminergic neurons in the alar plate of the caudal medulla. This neuronal population extends from the level of the IXth motor nucleus to the transition with the spinal cord, and they are mainly located ventromedial to the solitary tract. At the obex level, TH-positive cells are located in the outer aspect of the inner cell layer as a band oriented from dorsomedial to ventrolateral. TH-positive neurons have long processes directed horizontally or ventrolaterally to the dorsolateral funiculus. In double labeling (TH/NADPHd) experiments it was observed that the most ventrolaterally located neurons overlap with the NADPHd-positive neurons of the nucleus of the descending trigeminal tract. TH-positive processes of neurons of the nucleus of the solitary tract are also directed towards the dorsolateral funiculus and intermingle with the NADPHd-positive, dorsolaterally oriented processes of trigeminal neurons. Double TH/NOS-immunofluorescence experiments revealed results in line with that described for TH/NADPHd-staining.

Calbindin D-28K (Calb): In the caudal rhombencephalic alar plate two Calb-positive neuron populations can be distinguished (Figure 5). The first coincides with the nucleus of the solitary tract and it contains a band of neurons with processes towards the dorsolateral funiculus. The second is located in the dorsolateral gray and forms part of the nucleus of the descending trigeminal tract. Its cells have positive processes extending to the descending trigeminal tract. The highest density of both cell populations were observed at the obex level where the labeled processes of each population cross each other in their course to the dorsolateral funiculus and the descending trigeminal tract, respectively. Calb-positive neurons were not found in the DCN. In the dorsal horn of the spinal cord, however, an abundant Calb-positive cell population was observed that at cervical levels includes those cells of the caudal extent of the nucleus of the descending trigeminal tract.

Serotonergic (5-HT) and peptidergic labeling: Immunohistochemical labeling showed

that substance P-immunoreactive fibers are present in the dorsal aspect of the dorsolateral funiculus, more sparsely in the dorsal funiculus and almost no positive fibers are present in the descending trigeminal tract (Figure 7C,D). Some fibers leave the dorsolateral funiculus and may include primary afferents of the the tract of Lissauer and other non-primary projections, and innervate mainly the LCN area. Additionally, a few substance P-positive varicose fibers are present in the DCN, nucleus of the solitary tract and nucleus of the descending trigeminal tract. A similar pattern of Leu-enkephalin-immunoreactive terminal structures was found in the dorsal grey at obex levels in both the inner and the outer cell layers, including the DCN, LCN, nucleus of the solitary tract and nucleus of the descending trigeminal tract. These enkephalinergic fibers penetrate the gray from the dorsal funiculus, descending trigeminal tract and mainly from the dorsolateral funiculus. Neuropeptide Y-immunoreactive fibers enter the dorsolateral gray from the dorsal aspect of the lateral funiculus and form varicose fibers and terminal boutons. Serotonin-immunoreactive fibers innervate the DCN and LCN, and the adjacent structures of the inner cell layer including the nucleus of the solitary tract and nucleus of the descending trigeminal tract.

Tract-tracing experiments

In the previous sections evidence was provided for the presence of a DCN, an LCN, a nucleus of the descending trigeminal tract and a nucleus of the solitary tract within the ill-defined caudal rhombencephalic alar plate. In order to further characterize the DCN and the LCN of *Pleurodeles waltl*, the afferent and efferent connections of these structures were analyzed. To delineate the nucleus of the descending trigeminal tract, also the trigeminal innervation of the caudal rhombencephalon was studied.

Afferent connections

The primary afferent projections to the caudal rhombencephalic alar plate have been studied by applying BDA to cervical and lumbar spinal dorsal roots as well as to the trigeminal nerve root. Non-primary ascending projections from the spinal cord were studied by applying BDA to the dorsal horn of the cervical spinal cord.

Ascending dorsal root afferents.

Following the application of BDA to either brachial or lumbar dorsal roots, the labeled afferent fibers upon entering the spinal cord bifurcate into ascending and descending tracts. Two components can be distinguished in the spinal white matter: a medial bundle of thick fibers that enters the dorsal funiculus, and a more laterally located group of thin fibers within the dorsal portion of the dorsolateral funiculus, i.e. the tract of Lissauer. Both fiber systems project to widespread spinal and supraspinal regions in a manner that largely resembles that described for anurans (Antal et al., 1980; Székely et al., 1980; Nikundiwe et al., 1982a; Muñoz et al., 1996b). In the present study only the distribution of axons that innervate the caudal part of the rhombencephalic alar plate will be discussed. Ascending fibers in Lissauer's tract could be traced rostrally for only a few spinal segments giving off collateral branches that arborize and terminate in a ventral neuropil located at the lateral aspect of the deep dorsal and intermediate spinal grey. In the lumbar BDA experiments, ascending fibers in Lissauer's tract fade at thoracic levels and do not reach the obex region (Figure 6A). BDA applications to the second spinal dorsal root

labeled fibers in Lissauer's tract that ascend in a superficial position within the dorsolateral funiculus and reach the rostral pole of the IX-Xth cranial nerve root complex (Figure 6B). At upper cervical spinal levels and in the obex region a few branches leave the tract and turn medially to innervate the LCN in the outer cell layer and, to a lesser extent, the outer aspect of the inner cell layer where the caudal aspect of the nucleus of the descending trigeminal tract is located. More rostrally, Lissauer's tract courses ventrolaterally and some fibers leave the tract to arborize within the white matter adjacent to the reticular formation. The medial component is formed by ascending and descending dorsal funicular fibers that innervate the spinal gray of adjacent levels rostral and caudal to the entrance of the different roots. The rostrocaudal extent of this spinal projection exceeds that of Lissauer's tract. Most of the fibers arborize and form a terminal field in the dorsal aspect of the spinal gray, although some fibers continue ventrally to terminate within the central spinal gray.

Spinal primary afferents ascend to the obex region, somatotopically organized, within the dorsal funiculus. Fibers from lumbar dorsal roots occupy positions medial to those of cervical origin (Figure 6). These ascending spinal projections outline the DCN at the obex level (Figure A,B). The terminal fields in this area largely resemble the organization of the fibers in the dorsal funiculus. Thus, medially situated axons from lumbar dorsal root ganglion cells terminate on medial cells in the DCN, whereas laterally located fibers arising from cervical dorsal root ganglion cells end on lateral cells, with a certain degree of overlap in the projection. Most of the primary afferents terminate dorsal to the cells of the DCN. However, some varicose fibers extend more ventrolaterally and reach the area where the cells of the LCN are located. Rostral to the obex level, tightly packed dorsal funicular fibers gradually turn ventrolaterally and ascend throughout the medulla in a position dorsal to the descending trigeminal tract. Lumbar primary afferents hardly extend beyond the rostral limits of the DCN. Brachial primary afferents reach the cerebellum where they arborize profusely within the granule layer. Throughout the rhombencephalon, varicose fibers leave the tract and arborize within the white matter where dendrites of the adjacent periventricular cells of the reticular formation, nucleus of the solitary tract, nucleus of the descending trigeminal tract and the octavolateral area may be contacted.

Trigeminal afferents

After application of BDA to the proximal stump of a severed trigeminal nerve root, the primary trigeminal afferents were labeled (Figure 8). At the obex level, primary trigeminal fibers leave the descending trigeminal tract ventromedially and give off terminal branches to the outer and inner cell layers. These primary trigeminal branches are directed ventromedially, whereas dendrites of trigeminal neurons are directed dorsolaterally towards the descending trigeminal tract and they intermingle profusely (Figure 8C,D). Additionally, sparse trigeminal fibers reach the DCN, the nucleus of the solitary tract and the LCN.

Non-primary afferents

Spinal second-order projections also ascend via the dorsal funiculus and the dorsolateral funiculus. Tracer application to the cervical spinal cord revealed thin fibers coursing rostralwards throughout the rhombencephalon up to the cerebellum and the

subcerebellar region. These data are discussed in a separate paper (A. Muñoz et al., 1996b).

Efferent projections

Retrograde BDA tracing was used to further characterize the DCN and LCN with respect to their efferent projections. Experiments with tracer application to the ventral thalamus and to the torus semicircularis were done to label the cell populations in the caudal medulla and upper spinal cord that project to these targets of somatosensory projections.

Thalamic applications

In this group of experiments, BDA was applied to the thalamus. Although the tracer was applied mainly to the ventral thalamus, in some cases the dorsal thalamus was also affected. A distinct population of retrogradely labeled cells was observed, in the alar plate at the obex level composed of irregular and large cells together with round, small cells (Figure 9). Most of the cells were found contralateral to the injection site although a minor component of ipsilateral cells was also present. At the obex level, dorsomedially located neurons with processes directed dorsally into the dorsal funiculus, where spinal primary afferents ascend, were identified as the DCN. This neuronal population is formed by round, bipolar, irregular and oval shaped neurons that extend from medullary levels slightly rostral to the obex into the upper cervical spinal segment where they are tightly packed in the dorsalmost part of the gray. Additionally, ventrolaterally located neurons with dendritic processes directed ventrolaterally towards the dorsolateral funiculus were identified as the LCN. The LCN extends from the obex level rostrally to the cervical spinal cord, but extends more caudally than the DCN. LCN neurons are round, bipolar, oval shaped or irregular, and some of them are segregated from the gray within the dorsolateral funiculus proper. Although there is some degree of segregation between the DCN and the LCN, some labeled neurons were observed with processes directed both dorsally and ventrolaterally. The axons of both the DCN and LCN neurons could be traced into the contralateral medial lemniscus.

Toral applications

In five cases BDA was applied to the torus semicircularis. In some cases spread of the tracer to the mesencephalic dorsal tegmentum could not be avoided. In all cases neurons were retrogradely labeled within both the DCN and the LCN, but in higher numbers than in the cases of thalamic applications. They were more abundant on the contralateral side although a small ipsilateral component was also present. Dorsomedially located DCN cells possess several processes extending into the dorsal funiculus. Their axons mainly course ventromedially, cross the midline and form part of the contralateral medial lemniscus. LCN labeled cells are located in the lateral marginal zone of the dorsal grey or within the dorsolateral funiculus itself and extend more caudally than the DCN. LCN cells are large, bipolar or irregular cells with long processes directed mainly into the dorsolateral funiculus. Their axons could also be traced into the medial lemniscus.

Additionally, in both thalamic and toral BDA experiments retrogradely labeled neurons were observed in the dorsolateral part of the outer cell layer, thought to be part of the nucleus of the descending trigeminal nerve, since here trigeminal primary afferents arborize.

DISCUSSION

In the present study the organization, immunohistochemical characterization and the fiber connections of some of the neuronal components of the ill-defined alar gray at the spinomedullary transition level were investigated in the ribbed new, *Pleurodeles waltl*. More in particular, a DCN, an LCN, the nucleus of the solitary tract and the nucleus of the descending trigeminal tract which are hard to distinguish in Nissl-stained material, were (immuno)histochemically characterized. Additionally, the distribution of the spinal primary afferents from fore- and hindlimb-innervating spinal nerves, non-primary cervical spinal projections, the pattern of termination at the obex region of trigeminal primary afferents, and the organization of DCN and LCN neurons projecting to the thalamus and mesencephalon were studied with the BDA tract-tracing technique. In general, in *Pleurodeles waltl*, a similar pattern of organization of the various cell components of the alar plate at the spinomedullary transition level was found as in anurans (A. Muñoz et al., 1995, 1996a).

In cytoarchitectonic studies of the urodele brain stem no distinct DCN could be distinguished from the adjacent gray (Herrick, 1930, 1944, 1948; Kreht, 1940; Opdam and Nieuwenhuys, 1976). Herrick (1944) described a nucleus of the funiculus dorsalis in the alar plate of the obex region. Its boundaries with the nucleus commissuralis, i.e. the caudal continuation of the nucleus of the solitary tract, are not clear though its neurons are larger than those of the commissural nucleus. As far as we know, no LCN was described at the obex level or in the cervical spinal cord, and even controversial data are found in the literature concerning the existence of a cytoarchitectonically dictinct nucleus of the descending trigeminal tract in different species of urodeles (Herrick, 1930; Woodburne, 1936; Opdam and Nieuwenhuys, 1976; González and Muñoz, 1988). Herrick (1948) already noted the existence of the nucleus of the solitary tract in urodeles, and in a later study the nucleus of the solitary tract was described as a group of cells that surround the solitary tract throughout the medulla, although it could not be clearly delimited from the adjacent gray (Opdam and Nieuwenhuys, 1976).

The DCN has been considered as the site of termination of dorsal funicular fibers in the caudal brain stem, but not as a cytoarchitectonic entity (Nieuwenhuys and Cornelisz, 1971; Roth and Wake, 1985). Nevertheless, Nissl-stained sections of the brain stem at the obex level and of upper cervical levels allow the delineation of a DCN and even an LCN. Moreover, BDA labeling of spinal primary afferent projections passing via the dorsal funiculus to the obex level, labeling of non-primary ascending spinal projections at the dorsolateral funiculus (A. Muñoz et al., 1996b), and retrograde labeling from the thalamus and the torus semicircularis, clearly delineate the DCN and LCN in urodeles. Histochemical and immunohistochemical data are of great help in characterizing the nucleus of the solitary tract and nucleus of the descending trigeminal tract.

The NADPH-diaphorase (NADPHd) histochemical technique, known to stain specific neurons (Thomas and Pearse, 1964), can selectively stain particular populations of neurons in a Golgi-like manner (Scherer-Singler et al., 1983; Alonso et al., 1995; M. Muñoz et al., 1996). Throughout the brain, NADPHd and NOS localizations are identical (Bredt and Snyder, 1992). The distribution of NADPHd staining and neuronal NOS immunolabeling in the present study was also found identical. Therefore,

NADPHd can be used as a marker for NOS. Nitric oxide most likely plays a major role as a neuronal messenger (Bredt and Snyder, 1992; Meller and Gebhart, 1993; Schuman and Madison, 1994). The presence of NADPHd-positive cells and fibers in the mammalian spinal cord (Valtschanoff et al., 1992) suggests that nitric oxide may be involved in spinal sensory processing. In the rat DCN, Valtschanoff et al. (1993) found that most NOS-positive neurons are also immunoreactive for GABA, but not for the excitatory transmitters glutamate and aspartate. Moreover, since NOS-positive neurons could not be labeled retrogradely from the thalamus or spinal cord, they probably are local circuit neurons (Valtschanoff et al., 1993). In anurans, the presence of NADPHd-positive neurons in the alar plate at the obex level was recently described (A. Muñoz et al., 1995; M. Muñoz et al., 1996). NADPHd-positive neurons were found in the DCN, but more abundantly in the adjacent nucleus of the solitary tract and the nucleus of the tract of the trigeminal nerve in line with data in mammals (e.g., Leight et al., 1990; Vincent and Kimura, 1992; Dohrn et al., 1994; Takemura et al., 1994). In the present study in *Pleurodeles*, however, at the obex level labeling was restricted to neurons of the nucleus of the descending trigeminal tract. Only very few lightly stained cells were found more dorsally in the cell area that includes the DCN and the nucleus of the solitary tract. Weakly stained neurons were also found more rostrally in the nucleus of the solitary tract.

In mammals, calcium-binding proteins such as calbindin and parvalbumin show a preferential distribution for somatosensory structures including the DCN (e.g., Celio, 1990; Rausell and Jones, 1991a,b; Rausell et al., 1992; Menétrey et al., 1992a,b; Maslany et al., 1992; Ren and Ruda, 1994). Parvalbumin appears to be abundant in the pathway for epicritic sensibility, i.e. the dorsal column-medial lemniscal system, calbindin D-28k (Calb) occurs in the whole taste pathway of rats (Celio, 1990). In rats, Calb-positive neurons are found in certain laminae of the dorsal horn (see Antal et al., 1990; Ren and Ruda, 1994) including the cells of origin of ascending spinal projections (Menétrey et al., 1992b), in the sensory trigeminal nuclei as well as in the gracile and cuneate nuclei (Celio, 1990). Also in rats, Menétrey et al. (1992a) showed that Calb-positive neurons form a major part of the solitary and trigeminal projection systems. In monkeys, both proteins are differentially expressed in trigeminothalamic projections (Rausell and Jones, 1991a,b) and in spinothalamic projections (Rausell et al., 1992).

The presence of calbindin D-28k and parvalbumin in the alar plate of the spinomedullary transition area was recently demonstrated in the anuran brain (A. Muñoz et al., 1995). In line with these anuran data, in *Pleurodeles waltl*, Calb-positive neurons were found in the nucleus of the solitary tract and in the nucleus of the descending trigeminal tract, but not in the DCN or LCN. This pattern of distribution of Calb-positive neurons suggests that as in mammals, in amphibians, Calb positive cells are present in part of the somatosensory system including some neurons of the dorsal horn of the spinal cord and the nucleus of the trigeminal tract. In the anuran obex region, a distinct parvalbumin-positive cell population was found (A. Muñoz et al., 1995). Immunostaining for parvalbumin, rather clearly delineates the anuran DCN. However, with the same antibody and protocol no clear parvalbumin labeling, was found in the brain of *Pleurodeles*.

The caudal medullary and upper spinal alar plate in *Pleurodeles* is innervated by substance P-fibers

that course via the dorsal funiculus and mainly, the dorsolateral funiculus. Although all main regions, including the DCN, nucleus of the solitary tract and nucleus of the descending trigeminal tract are innervated, the LCN located at the dorsolateral aspect of the gray, in close relation to the dorsolateral funiculus receives the weakest innervation. These projections include primary spinal afferents in the dorsal funiculus and the tract of Lissauer according to Taban and Cathieni (1983) who described substace P-positive neurons in the spinal dorsal root ganglia, but may include other non-primary projections coursing in the dorsal funiculus and dorsolateral funiculus. In anurans, in which also CGRP-immunoreactive fibers was described (A. Muñoz et al., 1995), a similar innervation pattern exists (see also Adli et al., 1988; Petkó and Sánta, 1992). Additionally, this region is innervated by Leu-enkephalin, neuropeptide Y and serotonin-immunoreactive fibers resembling the situation in anurans (Ueda et al., 1984; Merchenthaler et al., 1989; Lázár et al., 1990; A. Muñoz et al., 1995). A similar peptidergic and serotoninergic innervation of the DCN is found in mammals (e.g., Steinbusch, 1981; Westman et al., 1984; Halliday et al., 1988; Blomqvist and Broman, 1993).

Urodele DCN: Cytoarchitecture and afferent connections

The urodele DCN was first described by Herrick (1944) as the nucleus of the funiculus dorsalis, located dorsolaterally to the nucleus commissuralis of Cajal from which it could not be clearly separated. Although in the axolotl a DCN was not considered a separated cytoarchitectonic entity (Opdam and Nieuwenhuys, 1976), Nissl-stained material of *Pleurodeles* revealed, in line with Herrick's (1944) observations that the DCN extends from the caudal rhombencephalic midvagal level up to the first cervical spinal segment. The rostral part of the DCN is composed of large neurons located dorsal to the solitary tract and is partially intermingled with smaller neurons of the caudal part of the lateral line area. At the obex level, the DCN is segregated at the dorsal aspect of the outer cell layer by a thin white matter layer. More caudally it occupies the most dorsomedial aspect of the spinal dorsal horn and the boundaries with the proper spinal neurons are hardly distinguishable. The urodele DCN is the main site of termination of spinal primary afferent fibers (Herrick, 1944; Nieuwenhuys and Cornelisz, 1971; Roth and Wake, 1985; the present study). As already noted in the axolotl (Nieuwenhuys and Cornelisz, 1971), in *Pleurodeles*, the medial component of the dorsal funiculus arises from lumbar ganglia and innervates the medial part of the DCN, whereas ascending fibers from cervical ganglia ascend in the lateral part of the dorsal funiculus and innervate the lateral aspect of the DCN. This somatotopic arrangement of dorsal column projections is comparable to that found in anurans (Antal et al., 1980; Nikundiwe et al., 1982; M. Muñoz et al., 1991; A. Muñoz et al., 1995). However, in contrast to anurans, in *Pleurodeles*, medial, "gracile" and lateral, "cuneate" components of the DCN can not be recogniced, neither cytoarchitectonically nor by means of retrograde tracing from the thalamus or the torus semicircularis.

Dorsal funicular fibers in *Pleurodeles* continue rostrally to the obex level and innervate the nucleus of the solitary tract, reticular formation, the nucleus of the descending trigeminal tract as well as the vestibular nuclear complex, and reach the granule layer of the cerebellum. Apart from spinal primary afferents, the presence of non-primary spinal fibers to the DCN or postsynaptic dorsal column system

(PDCS) is suggested for urodeles (A. Muñoz et al., 1996b). The presence of a somatotopically arranged PDCS projection was demonstrated in anurans (ten Donkelaar and de Boer-van Huizen, 1991; A. Muñoz et al., 1995) and throughout other terrestrial vertebrates (e.g., Rustioni, 1973; Angaut-Petit, 1975a, b; Uddenberg, 1968; Rustioni and Kaufman, 1977; Bennett et al., 1984; Giesler et al., 1984; Funke, 1988; Pritz and Stritzel, 1994).

Some fibers of the descending trigeminal tract, including cutaneous fibers from the Vth, VIIth and IXth cranial nerves also reach the DCN in *Pleurodeles* (González and Muñoz, 1987; A. Muñoz et al., 1995). Additionally, dorsolateral funicular fibers, some of them substance-P-, Leu-Enk-, or NPY-positive, five off collateral branches, and innervate the LCN and the more dorsomedially located targets i.e. the nucleus of the descending trigeminal tract and the DCN.

Urodele LCN: Cytoarchitecture and afferent connections

The LCN in urodeles was not noticed in previous studies (Herrick, 1944, 1948; Opdam and Nieuwenhuys, 1976). In *Pleurodeles* the LCN, is not a clearly segregated cell mass in the ventrolateral aspect of the outer cell layer at the spinomedullary transition level, but it can be identified by means of immunohistochemical and hodological studies, as also shown for anurans (A. Muñoz et al., 1996a). Some of the LCN neurons are segregated into the dorsolateral funiculus. LCN afferents include spinal primary afferents of Lissauer's tract, at least from brachial dorsal roots, and non-primary spinal projections from the spinocervical tract. Some of the LCN afferents are immunopositive for substance-P, Leu-Enk, NPY and serotinin-positive. Additionally, the LCN cells probably receive a trigeminal primary afferent input (González and Muñoz, 1988; the present study). A similar immunohistochemical characterization of afferent terminals upon cells of the LCN was recently described for anurans (A. Muñoz et al., 1995).

Urodele DCN and LCN: Efferent connections

Herrick (1944, 1948; see also Herrick and Bishop, 1958) denied the existence of a medial lemniscus in *Amblystoma tigrinum*, and compared the nucleus of the funiculus dorsalis in urodeles with the external cuneate nucleus and not with the gracile and cuneate nuclei of mammals. Herrick suggested that proprioceptive arcuate fibers, that arise at the region of the calamus, including the nucleus of the funiculus dorsalis, join the dorsal component of the spinal lemniscus where they ascend, ventral to the descending trigeminal tract to innervate the medullary and midbrain reticular formation, the tectum and the dorsal (sensory) thalamus. Additionally, Herrick (1944, 1948; see also Herrick and Bishop, 1958) reported the existence of the general bulbar lemniscus as a crossed fiber system that arises from the nucleus of the funiculus dorsalis (exteroceptive fibers), the spinal nucleus of the trigeminal nerve and other medullary centers and ascend at the ventrolaterally white matter of the medulla to innervate the reticular formation, the tectum mesencephali and also the dorsal (sensory) thalamus, overlapping with those projections of the spinal lemniscus. However, the present study clearly demonstrates that the 'nucleus' identified in *Pleurodeles waltl* is comparable to the dorsal column nuclei of anurans and other vertebrates, which together with the LCN, give rise to the medial lemniscus. Herrick (1944, 1948) also described ipsilateral fibers from the nucleus of the funiculus dorsalis that would reach the

cerbellum through the tracts A and B of Kingsbury (1895), that would have different function from those fibers from the dorsal funicular nucleus that reach the cerebellum through the spinal lemniscus. Although in anurans no equivalents of the urodele tracts A and B have been described it is noteworthy that an ipsilateral cerebellar projection from the DCN ascending dorsal to the descending trigeminal tract together with the spinal primary afferents, was demonstrated in *Rana perezi* and in *Xenopus laevis* (González et al., 1984; A, Muñoz et al., 1995).

In the present study the existence in urodeles of medial lemniscal projections from the DCN and the LCN to the ventral thalamus and the torus semicircularis was demonstrated. Axons of DCN and LCN projection neurons cross the midline ventral to the caudalmost aspect of the fourth ventricle or the central canal to join the contralateral medial lemniscus where they ascend towards the torus semicircularis and the thalamus. In urodeles and in anurans a convergence of spinal (A. Muñoz et al., 1994a, 1996b) and medial lemniscal (DCN and LCN) inputs (Silvey et al., 1974; Neary and Wilczynski, 1977, 1979; Forehand and Farel, 1982; Urbán and Székely, 1982; A. Muñoz et al., 1994b, 1995, 1996a) in the torus semicircularis was demonstrated. A similar convergence appears in the ventral thalamus. The torus semicircularis of urodeles is not clearly distinguishable in silver or Nissl-stained sections (Herrick, 1942; Opdam and Nieuwenhuys, 1976), although it was proposed as a center for integration of lateral line and vestibular information (Herrick, 1948). However, in later studies the torus semicircularis was unequivocally delineated (Roth et al., 1990) based on its afferent connections (González and Muñoz, 1987; Manteuffel and Naujoks-Manteuffel, 1990), its descending medullary and spinal projections (Naujoks-Manteuffel and Manteuffel, 1988) and by registration of visual, vibration and auditory responses (Manteuffel and Naujoks-Manteuffel, 1990). In line with our data on the medial lemniscal (DCN and LCN) innervation of the torus semicircularis in *Pleurodeles*, Manteuffel and Naujoks-Manteuffel (1990) found retrogradely labeled neurons bilaterally in the dorsal periventricular gray of the most caudal part of the medulla oblongata. Although convergence of somatosensory spinal (A. Muñoz et al., 1996b) and medial lemniscal inputs including DCN/LCN (the present study) seems to exist in the urodele torus semicircularis, detailed physiological studies are needed in order to establish the somatotopic representation of the contralateral body surface as found in anurans (Comer and Grobstein, 1981).

Apart from the thalamus and the torus semicircularis, it seems likely (unpublished observations) that the medial lemniscus in urodeles includes fibers that reach the posterior tubercle, the mesencephalic optic tectum, mesencephalic tegmental nuclei, the isthmic region, the cerebellum, the area octavolateralis and the rhombencephalic reticular formation in line with data in anurans (A. Muñoz et al., 1995).

Various studies have dealt with somatosensory processing in the deep neuropil and the gray of the optic tectum of different urodele species (Grüsser-Cornehls and Himstedt, 1973; Gruberg and Solish, 1978; Gruberg and Harris, 1981; Harris, 1982, 1989; Stirling and Brändle, 1982). Roth et al. (1990) described the tectum as a polysensory integrative center of retinal and non-retinal afferents. Their cell types 1 and 2c are probably involved in the processing of somatosensory information. Gruberg and Solish (1978) observed a rostrocaudal and lateromedial somatosensory body representation in layer 3 of the tectum formed by

contralateral and deeper bilateral somatosensory proccessing units. In various studies a spinal innervation of the urodele tectum mesencephali was shown, although some controversies with regard to the cells of origin of this projection exist (Herrick, 1914, 1942, 1948; Nieuwenhuys and Cornelisz, 1971; Jakway and Riss, 1972; Gruberg, 1972; Finkenstädt et al., 1983; Rettig, 1988; A. Muñoz et al., 1996b). The urodele tectum may also receive somatosensory information from the DCN/LCN via the medial lemniscus as demonstrated in *Xenopus laevis* (A. Muñoz et al., 1995). Until now, the latter pathway has not been unequivocally demonstrated for urodeles in studies on the cells of origin of non-retinal tectal afferents (Rettig, 1988, Finkenstädt et al., 1983). After HRP injections in the tectum, Finkenstädt et al. (1983) labeled cells in the upper cervical spinal cord in positions corresponding to the DCN or LCN, as observed in the present study in *Pleurodeles*.

CONCLUSIONS

In the caudal rhombencephalic alar plate of the ribbed newt, *Pleurodeles waltl*, a DCN and an LCN can be distinguished lateral to an inner periventricular zone composed of the nucleus of the solitary tract and the nucleus of the descending trigeminal tract. NADPH-diaphorase histochemistry and calbindin D-28k immunohistochemistry stain only neurons in the inner compact cell layer. Tracing experiments showed that the dorsal column is somatotopically arranged: lumbar primary afferents terminate on medial DCN neurons, whereas cervical primary afferents terminate on lateral DCN neurons. The LCN is densely innervated by the spinocervical tract present in the dorsolateral funiculus. Retrograde tracing showed extensive predominantly contralateral projections of both the DCN and LCN to the torus semicircularis and the ventral thalamus.

These data show that in urodeles a dorsal column-medial lemniscus system via the DCN as well as a spinocervicothalamic system via the LCN are present.

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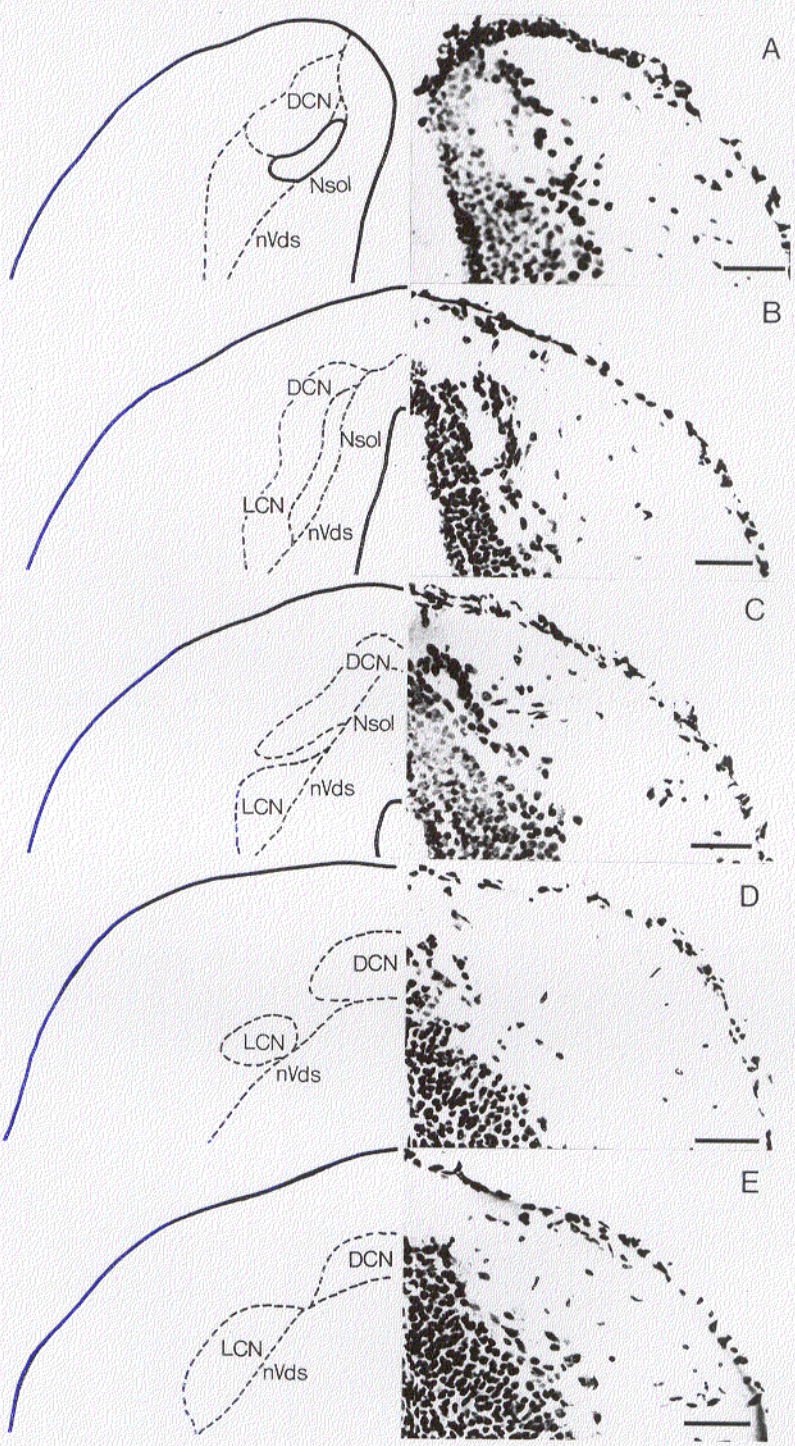

Figure 1: Photomicrographs of Nissl-stained transverse sections and schematic drawings of the caudal part of the rhombencephalic alar plate in *Pleurodeles waltl*. Scale bars indicate 100 µm.

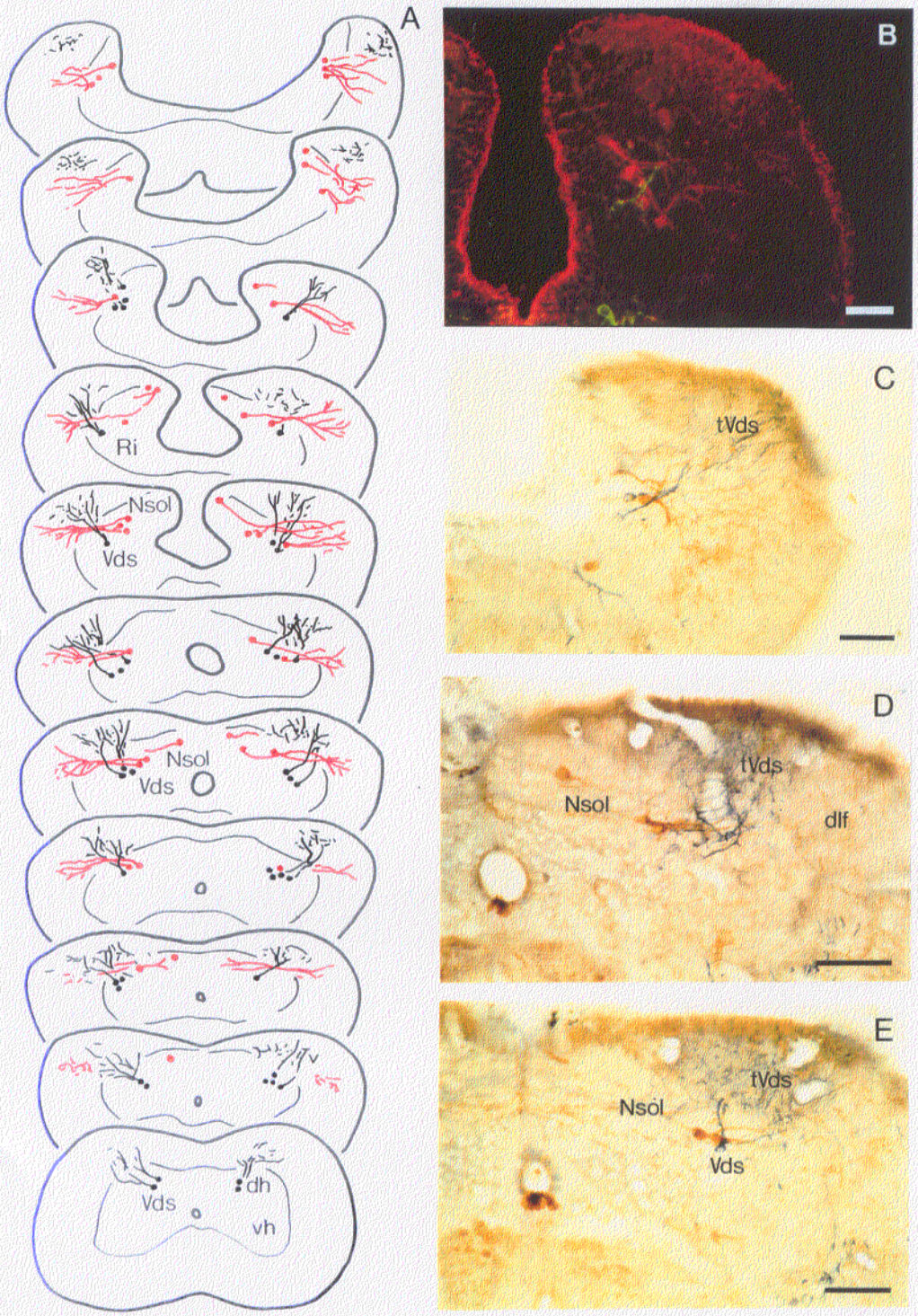

Figure 2: A, The distribution of NADPH diaphorase-positive (black) and TH (red) cells in the caudal part of the rhombencephalic alar plate of *Pleurodeles waltl.* C-E, Photomicrographs illustrating examples of NADPH diaphorase-positive (blue) and TH (brown) positive cells. B, an example of the NOS (green) and TH (red)-positive neurons in the alar plate at the obex region. Scale bars indicate 100 µm.

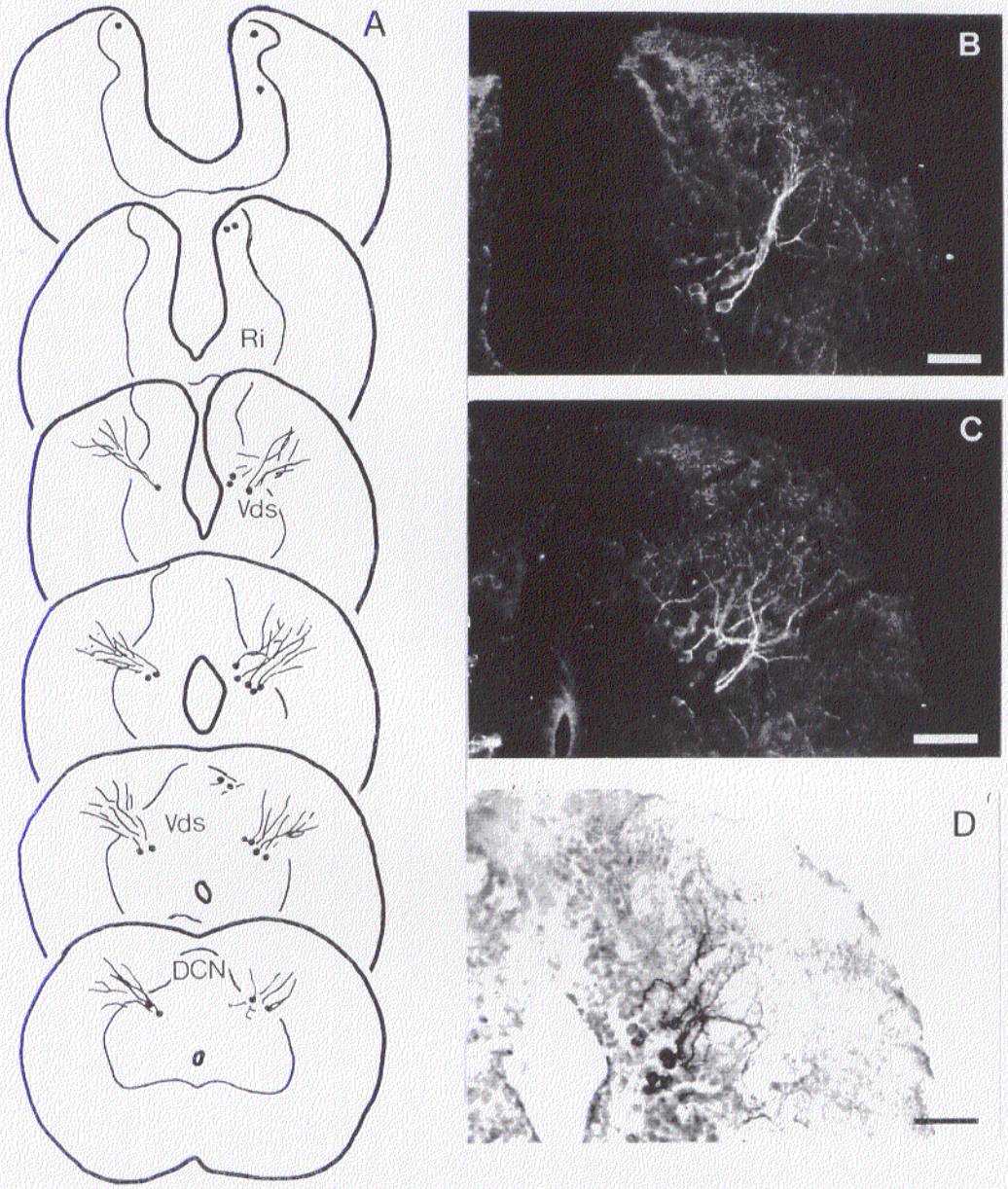

Figure 3: The distribution of NOS-positive neurons in the caudal part of the rhombencephalic alar plate and the rostral spinal cord of *Pleurodeles waltl*. Photomicrographs show examples of the labeling observed in the nucleus of the descending trigeminal tract. Scale bars indicate 100 µm.

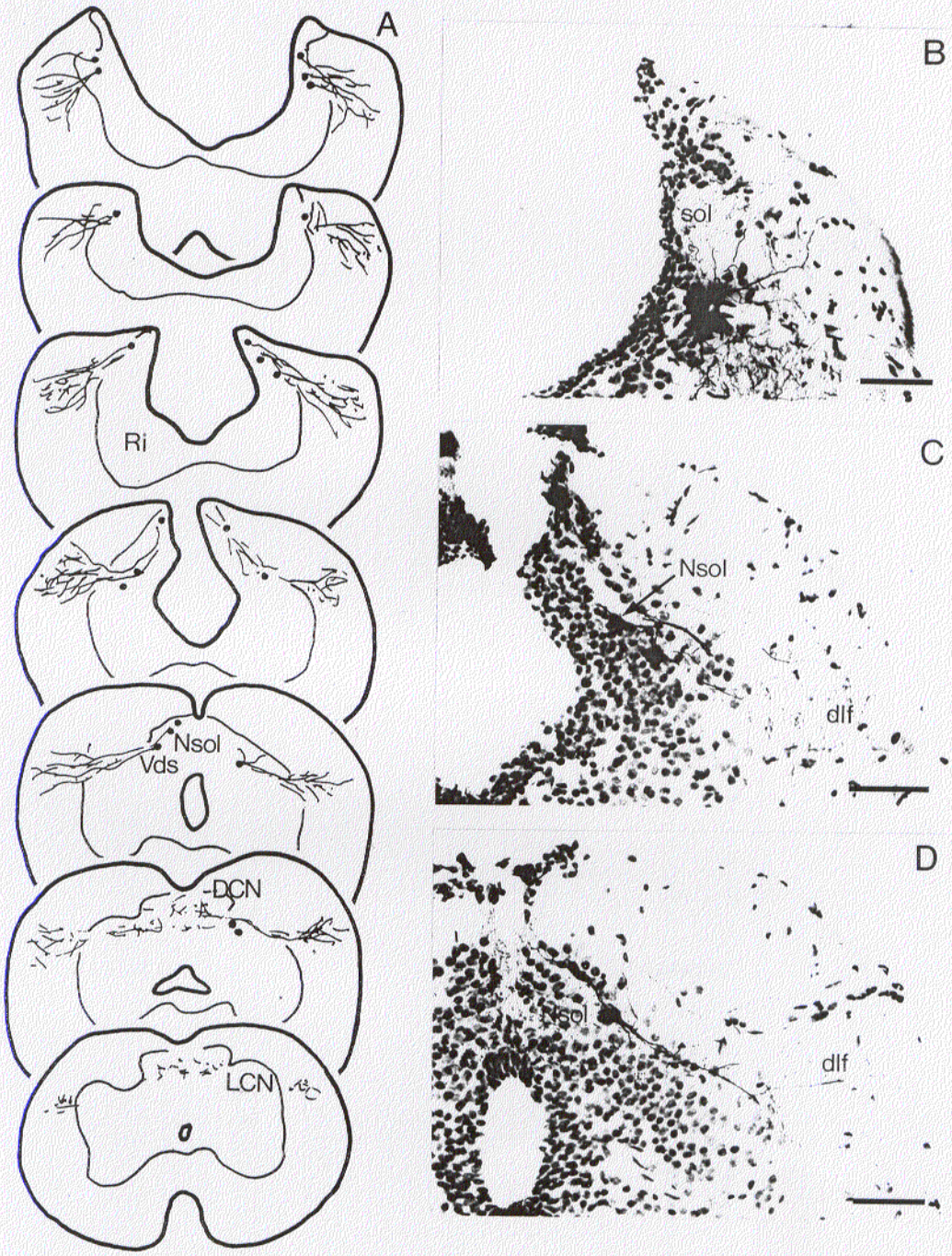

Figure 4: A, The distribution of TH-positive neurons in the caudal part of the rhombencephalon and the most rostral part of the spinal cord of *Pleurodeles waltl.* B-D, Photomicrographs of examples of TH labeling in the nucleus of the solitary tract. Scale bars indicate 100 µm.

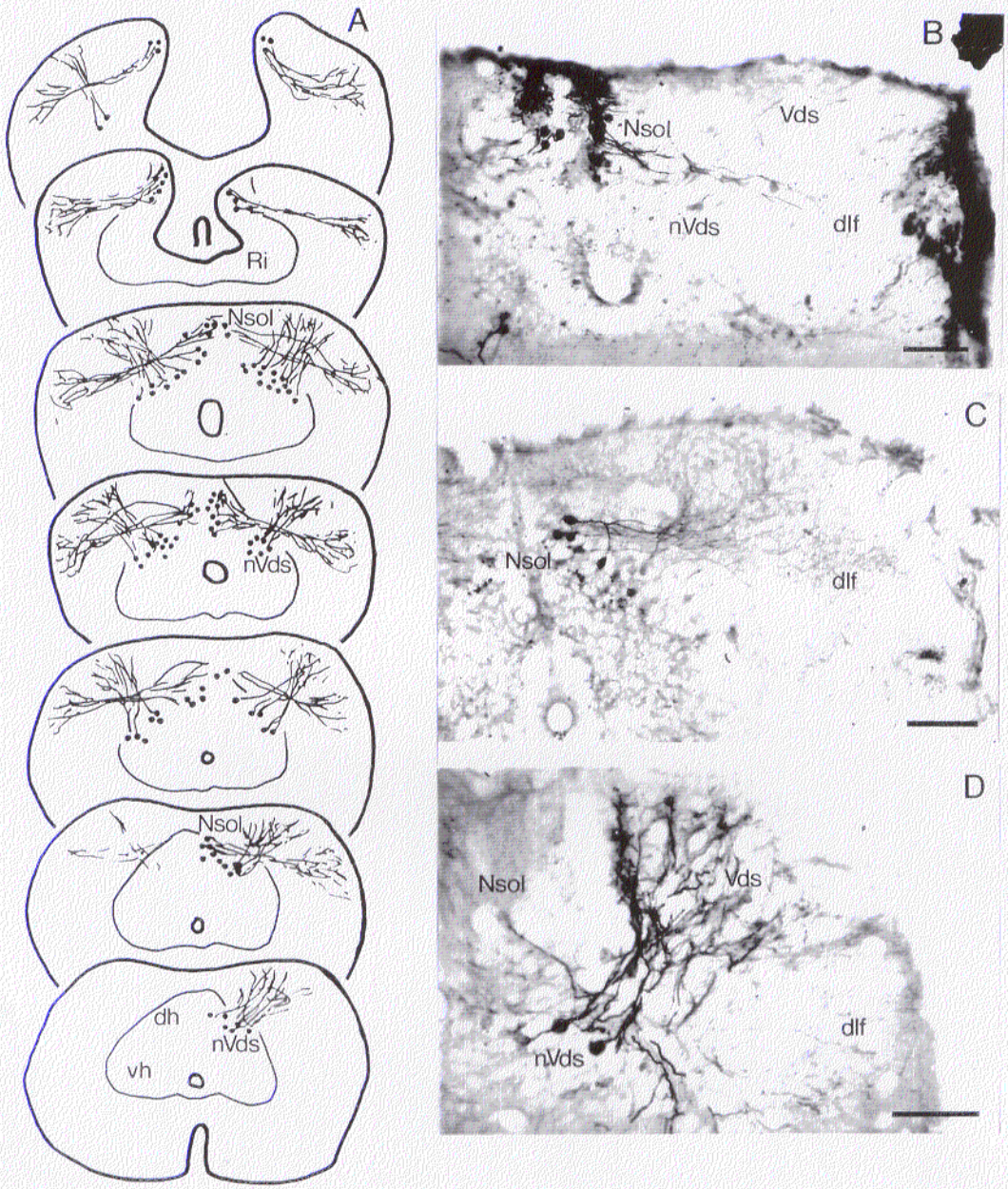

Figure 5: The distribution of calbindin D-28k-positive neurons in the caudal part of the rhombencephalic alar plate and the rostral spinal cord of *Pleurodeles waltl*. Photomicrographs show examples of the labeling observed in the nucleus of the solitary tract and in the nucleus of the descending trigeminal tract. Scale bars indicate 100 µm.

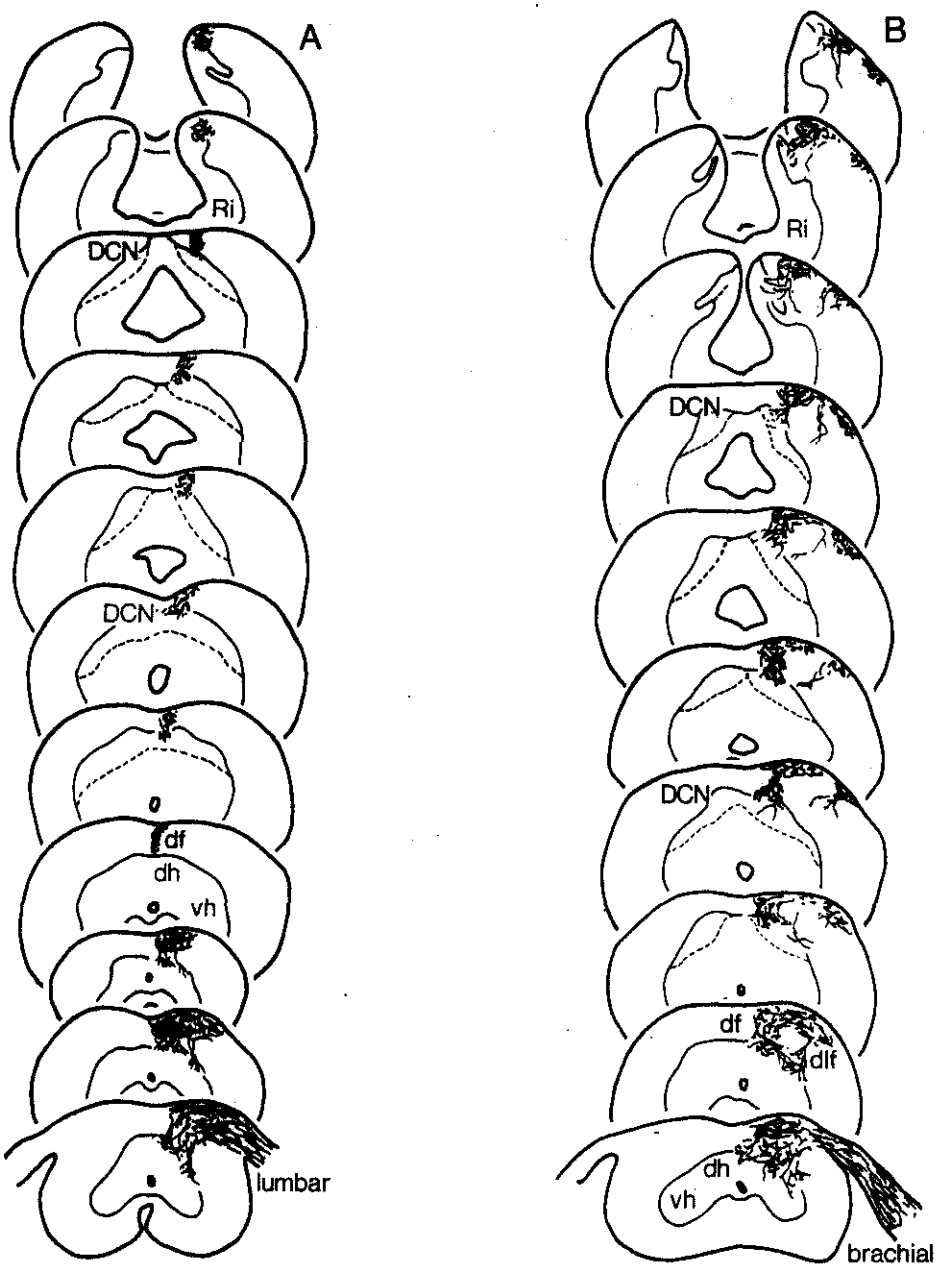

Figure 6: Schematic drawings of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodeles waltl* showing the distribution of lumbar (A) and brachial (B) dorsal root afferents.

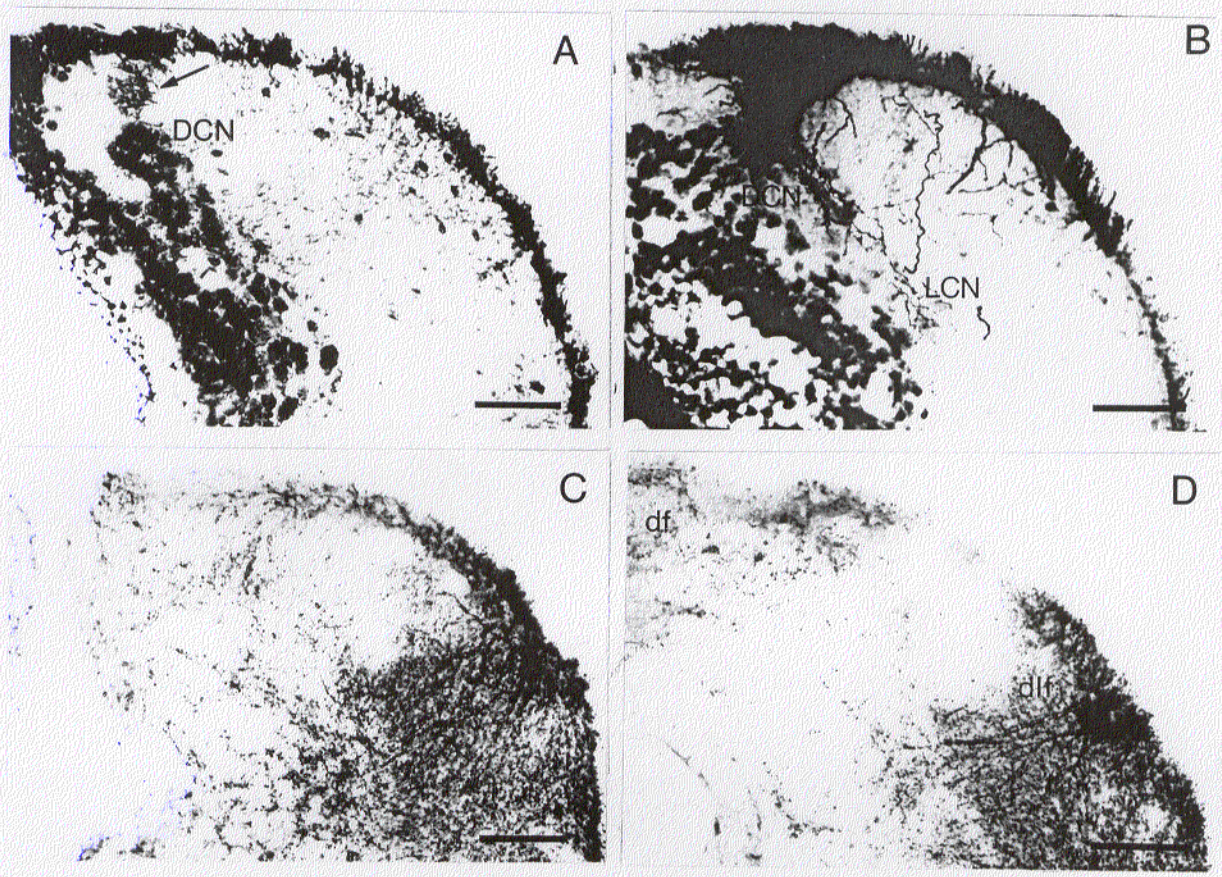

Figure 7: A, B, Photomicrograph showing the termination pattern of BDA labeled brachial and lumbar dorsal root afferents at the rostral DCN of *Pleurodeles waltl*. Scale bars indicate 100 µm. C,D, Photomicrographs showing the substance P-immunoreactive innervation of the caudal medullary (C) and rostral spinal (D) gray. Scale bars indicate 100 µm.

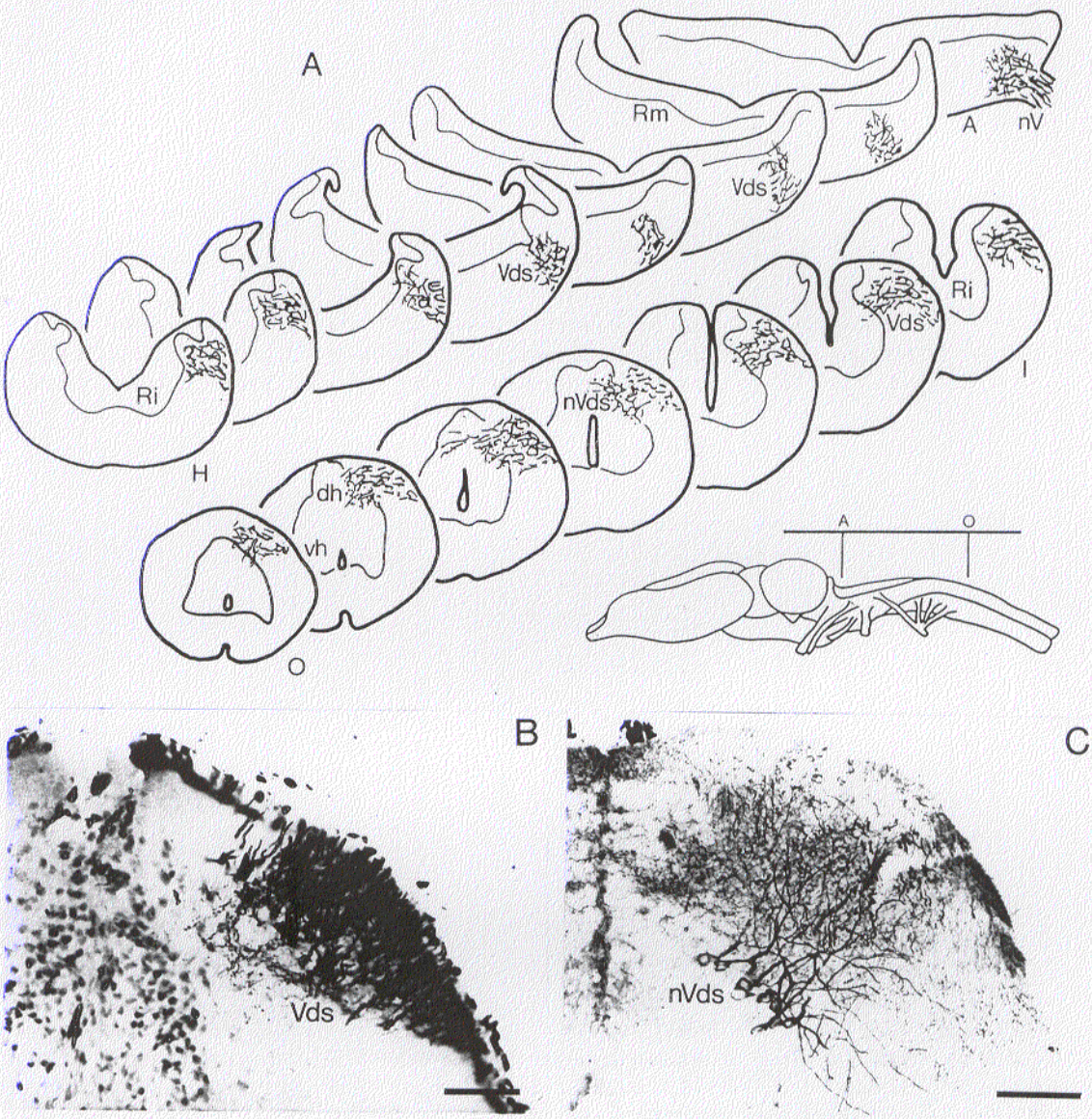

Figure 8: Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodesle waltl* showing the distribution of trigeminal afferent fibers. B, Photomicrograph showing ipsilateral trigeminal afferents to the obex region. C, Photomicrograph showing NAHPHd positive neurons in the nucleus of the descending trigeminal tract with dendrites directed to the Vds.

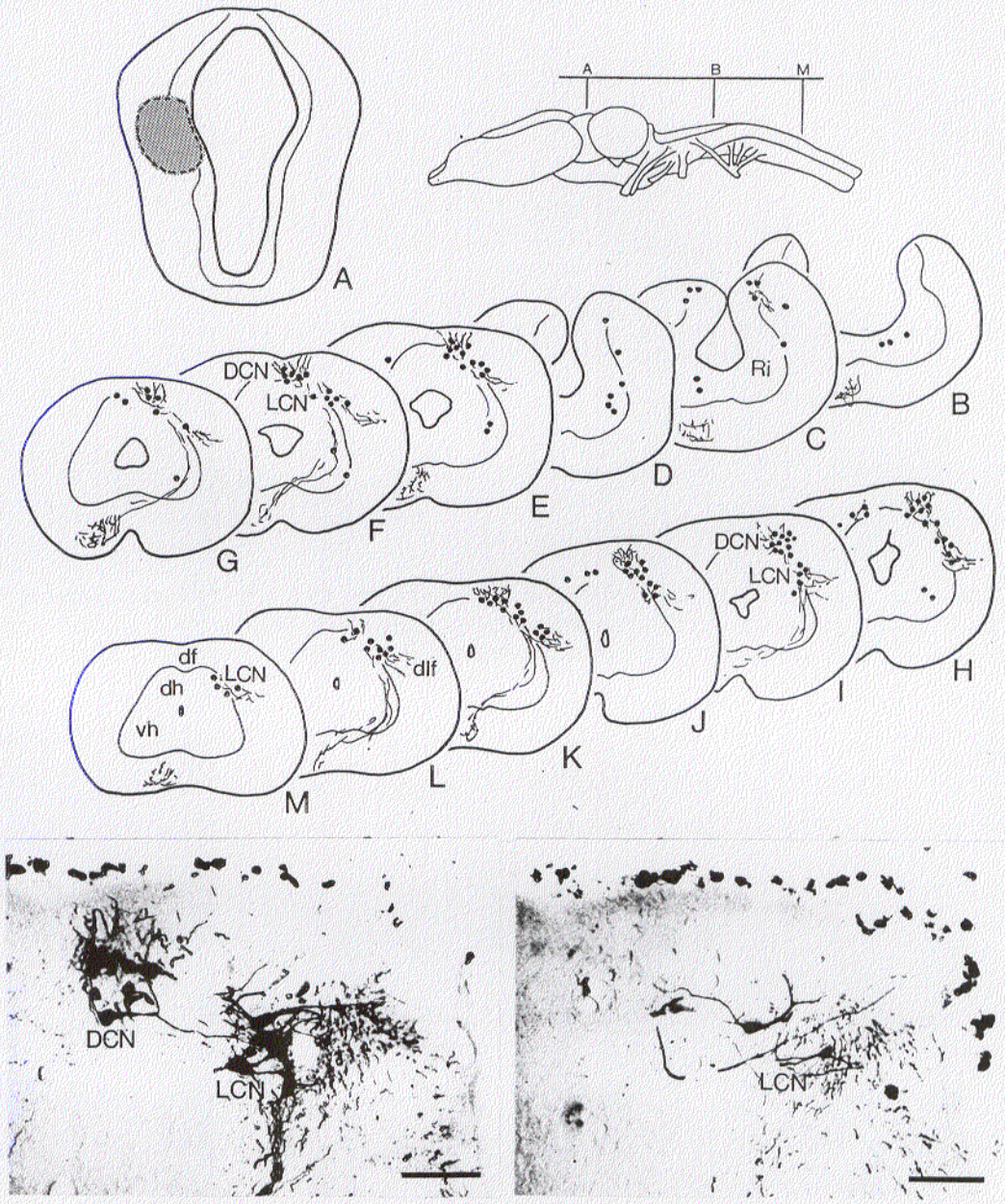

Figure 9: Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodeles waltl* the distribution of retrogradely labeled neurons following a BDA application to the thalamus. Examples of labeling are shown in two photomicrographs. Scale bars indicate 100 µm.

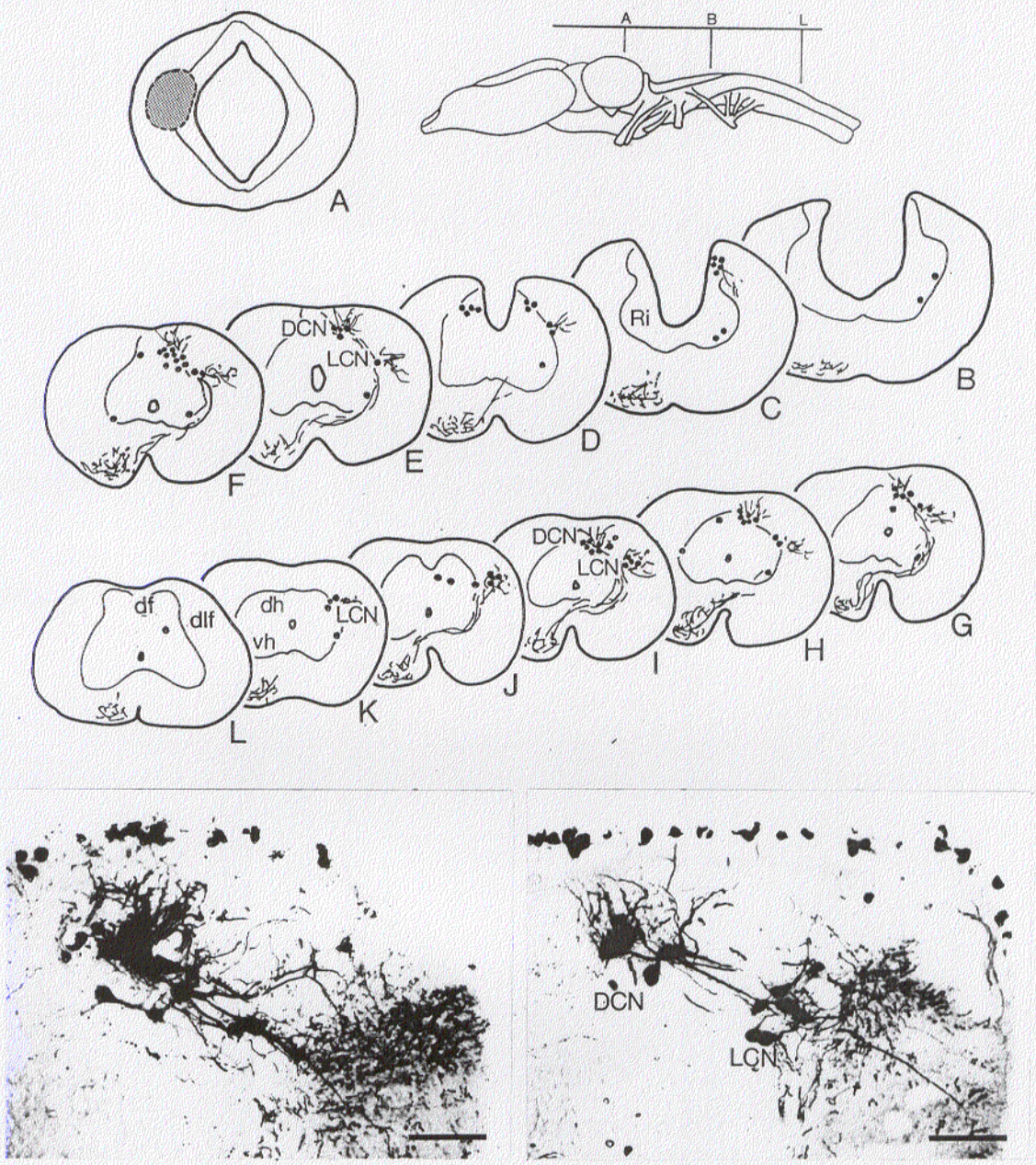

Figure 10: Schematic drawing of a series of transverse sections through the brainstem and rostral spinal cord of *Pleurodeles waltl* showing the distribution of retrogradely neurons following a BDA application to the torus semicircularis. Examples of labeling are shown in two photomicrographs. Scale bars indicate 100 µm.

ABBREVIATIONS

DCN	dorsal column nucleus
df	dorsal funiculus
dh	dorsal horn
dlf	dorsolateral funiculus
LCN	lateral cervical nucleus
Nsol	nucleus of the solitary tract
nV	trigeminal nerve
nVds	nucleus of the descending tract of the trigeminal nerve
Ri	nucleus reticularis inferior
Vds	descending tract of the trigeminal nerve
vh	ventral horn

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Organization of the caudal rhombencephalic alar plate of the ribbed newt Pleurodeles waltl: Evidence for the presence of dorsal column and lateral cervical nuclei

COMENTARIOS

6.2

El lemnisco medial, formado en parte por las proyecciones ascendentes procedentes de los núcleos de la columna dorsal y cervical lateral, constituye uno de los principales sistemas ascendentes de transmisión de información somatosensorial a centros supraespinales, en vertebrados terrestres. Los núcleos de la columna dorsal reciben aferencias espinales primarias y no primarias, a través del funículo dorsal, mientras que el núcleo cervical lateral se encuentra inervado por el tracto espinocervical que asciende por el funículo dorsolateral (Willis y Coggeshall, 1991). En anuros se ha demostrado recientemente la presencia del núcleo de la columna dorsal (DCN) y cervical lateral (LCN) en la placa alar de la región del óbex, así como del lemnisco medial (capítulo 5 de la presente memoria). Sin embargo, en urodelos no existen hasta el momento evidencias experimentales que demuestren la presencia de este sistema.

El sistema nervioso central de urodelos se ha considerado menos evolucionado que el de otros anfibios, debido a su escasa diferenciación citoarquitectónica, y emigración de las masas celulares, que, por ejemplo en el tronco cerebral (Opdam y Nieuwenhuys, 1976) se disponen, densamente agrupadas, en una zona continua de sustancia gris periventricular. Herrick (1948), sin embargo, sugirió que dentro de la simple disposición de neuronas y fibras, el cerebro de la salamandra *Ambystoma tigrinum* presenta una organización similar a la de otros vertebrados. Estudios posteriores han demostrado que al menos algunos sistemas del cerebro de urodelos presentan características anatómicas y funcionales similares a las de anuros, lo que conduce a un patrón de comportamiento comparable (Roth, 1987, Roth y cols., 1993), por lo que se ha sugerido una *simplificación secundaria* (Northcutt, 1987; Roth y cols., 1992, 1993), según la cual el cerebro de este grupo de vertebrados aparenta ser más primitivo de lo que cabría esperar, según su posición filogénica (Roth y cols., 1992).

Citoarquitectura

En el presente estudio se han caracterizado, en el urodelo *Pleurodeles waltl*, los distintos componentes neuronales relacionados con el procesamiento de información somática, presentes en la placa alar de la región del óbex, citoarquitectónica y quimioarquitectónicamente, así como su conectividad.

Debido a la escasa diferenciación en estudios citoarquitectónicos en urodelos (Herrick, 1930, 1944, 1948; Kreht, 1940; Opdam y Nieuwenhuys, 1976) no se han descrito el DCN ni el núcleo del tracto solitario (Nsol), como centros diferenciables de las masas celulares adyacentes, y existen datos controvertidos en cuanto a la presencia del núcleo del tracto descendente del nervio trigémino (nVds) como entidad citoarquitectónica (Herrick, 1930; Woodburne, 1936, Opdam y Nieuwenhuys, 1976; González y Muñoz, 1988). Herrick (1944), en *Ambystoma tigrinum*, en estudios con ténicas de Golgi sugirió la presencia del *nucleus funiculus dorsalis*, en la placa alar de la región del óbex, relacionado con fibras del funículo dorsal y en menor grado con fibras vestibulares, trigeminales y de la línea lateral; escasamente segregado del *nucleus comisuralis*, el cual representa la prolongación caudal del núcleo del tracto solitario y recibe aferencias viscerales del tracto solitario. La presencia del DCN ha sido considerada en estudios posteriores, en base al patrón de terminación de las aferencias primarias espinales en esta región (Nieuwenhuys y Cornelisz, 1971; Roth y Wake, 1985). Sin embargo, hasta el momento en uodelos no se ha sugerido la existencia del LCN en niveles de transición espinorombencefálicos.

En el presente trabajo se han identificado en *Pleurodeles waltl*, el DCN y el LCN en la placa alar a nivel del óbex mediante técnicas de Nissl. En dicha región hemos observado, además de una *banda celular interna* periventricular, gruesa y con células densamente empaquetadas, una *banda celular externa*, delgada y separada de la anterior por una estrecha lámina de sustancia blanca. El DCN consiste en una agrupación celular localizada en la región dorsomedial de la banda celular externa, en relación con la sustancia blanca del funículo dorsal. Dicho núcleo se extiende desde los primeros segmentos espinales rostrales hasta niveles del núcleo motor del nervio vago, y en él no se distingue una segregación mediolateral de los componentes *gracilis* y *cuneatus*. El LCN, situado ventrolateralmente en la banda celular externa, está formado por neuronas, agrupadas menos densamente que en el caso del DCN, que se entremezclan con la sustancia blanca del funículo dorsolateral, y se extiende desde el óbex hasta los primeros segmentos espinales. Esta organización citoarquitectónica ha sido corroborada con experimentos inmunohistoquímicos y mediante el estudio de las conexiones aferentes y eferentes de ambos núcleos (ver más adelante).

El Nsol y el nVds, identificados en base a resultados hodológicos e inmunohistoquímicos, se localizan en la banda celular interna en posiciones dorsomedial y ventrolateral respectivamente, si bien no están citoarquitectónicamente segregados entre si, ni de otras celulas ventromediales a ellos, por lo que no pudieron ser identificados mediante tinciones de Nissl.

Quimioarquitectura

En mamíferos la sintasa del óxido nítrico (NOS), el cual probablemente desempeña un papel importante como mensajero neuronal (Bredt y Snyder, 1992; Meller y Gebhart, 1993; Schuman y Madison, 1994), se expresa en neuronas de núcleos relacionados con el procesamiento de información somática, como los núcleos de la columna dorsal, el nVds y el Nsol, presentes en la placa alar de la región del óbex (Leight y cols., 1990; Vincent y Kimura, 1992; Valtschanoff y cols., 1993; Dohrn y cols., 1994; Takemura y cols., 1994). Debido a que la NOS tiene actividad NADPH-d (Dawson et al., 1991; Hope et al., 1991), la distribución de ambas son idénticas (Bredt y Snyder, 1992), por lo que la NADPHd puede usarse como un marcador para NOS.

En nuestros experimentos en *Pleurodeles waltl*, con tinciones tanto histoquímicas frente a NADPHd como inmunohistoquímicos frente a NOS, hemos observado una población muy patente de neuronas positivas en la región del óbex, localizadas en la mitad externa y ventrolateralmente dentro de la banda celular interna. Las dendritas de estas células se dirigen dorsolateralmente e invaden la sustancia blanca dorsolateral, correspondiente al tracto descendente del nervio trigémino (Vds), por lo que dicha población neuronal se ha considerado como una parte del nVds, coincidiendo con los datos descritos en mamíferos (Leight y cols., el 1990; Vincent y Kimura, 1992; Dohrn y cols., 1994; Takemura y cols., 1994).

En *Pleurodeles waltl* únicamente hemos observado un escaso número de neuronas positivas para NADPHd o NOS en el DCN o el Nsol a nivel de óbex, contrastando con los datos descritos en anuros (A. Muñoz y cols., 1995: M. Muñoz y cols., 1996) y en mamíferos (Leight y cols., 1990; Vincent y Kimura, 1992; Valtschanoff y cols., 1993; Dohrn y cols, 1994; Takemura y cols., 1994). La mayor concentración de neuronas positivas para NADPHd o NOS en el Nsol está presente en los niveles rombencefálicos más

rostrales. En experimentos en los que se combinaron las tinciones para NADPHd o NOS con la inmunodetección de la tirosina hydroxilasa (TH), se comprobó que la población neuronal positiva para NADPHd-NOS en estos niveles, se dispone ventralmente al tracto solitario y está entremezclada con neuronas catecolaminérgicas (positivas para TH) del Nsol, por lo que consideramos que pertenece a dicho núcleo. En la región del óbex las neuronas catecolaminérgicas del Nsol se disponen en la zona dorsomedial de la banda celular interna, y sus prolongaciones se dirigen lateral y ventrolateralmente hasta el funículo dorsolateral. En su recorrido dichas prolongaciones se cruzan con las dendritas de las neuronas del nVds, positivas para NOS-NADPHd y dirigidas hacia el Vds.

En mamíferos la proteína ligante de calcio, calbindina D28k (Calb), se expresa en algunas estructuras somatosensoriales (Celio, 1990; Rausell y Jones, 1991a,b; Rausell y cols., 1992; Menétrey y cols., 1992a,b; Maslany y cols., 1992; Ren y Ruda, 1994). En la rata, existen neuronas positivas para Calb en determinadas láminas del asta dorsal de la médula espinal (Antal y cols., 1990; Menétrey y cols., 1992b; Ren y Ruda, 1994), en los núcleos sensitivos trigeminales y en menor número en los núcleos *gracilis* y *cuneatus* (Celio, 1990; Maslany y cols., 1992). En distintas especies de mamíferos se ha demostrado que las neuronas positivas para Calb, constituyen una parte importante de los sistemas de proyección trigeminales, del núcleo del tracto solitario y de las proyecciones espinales ascendentes (Rausell y Jones, 1991a,b; Rausell y cols., 1992; Menétrey y cols., 1992a).

Nuestros resultados en *Pleurodeles waltl*, de acuerdo con las observaciones realizadas en anuros (A. Muñoz y cols., 1995), demuestran la existencia de dos poblaciones neuronales positivas para Calb en la placa alar de la región del óbex. Se localizan dorsomedial y ventrolateralmente en la banda celular interna y pertenecen al Nsol y nVds respectivamente. Las prolongaciones de las neuronas positivas para Calb del Nsol y nVds se entremezclan en sus recorridos hacia el DLF y el Vds respectivamente.

En experimentos inmunohistoquímicos hemos podido comprobar la presencia de fibras inmunoreactivas para sustancia P y Leu-encefalina, en el DLF y en menor grado en el DF y el Vds. Igualmente se observó que existen terminales positivos de sustancia P y Leu-encefalina, así como para serotonina, que alcanzan el DCN, LCN, Nsol y nVds. El patrón de marcaje para neuropéptido Y en esta región es más restringido y se limita a fibras positivas que abandonan el DLF, para inervar la región lateral de la placa alar. Esta inervación peptidérgica y serotoninérgica está de acuerdo con los datos obtenidos en anuros (capítulo 5 de la presente memoria; Ueda y cols., 1984; Adli y cols., 1988; Merchenthaler y cols., 1989; Lázár y cols., 1990; Petkó y Sánta, 1992; A. Muñoz y cols., 1995) y con resultados descritos en mamíferos (Steinbusch, 1981; Westman y cols., 1984; Halliday y cols., 1988; Ibuki y cols., 1989; Tamatani y cols., 1989; Conti y cols., 1990; Fabri y Conti, 1990; Blomqvist y Broman, 1993).

Conectividad

Además de los criterios citoarquitectónicos e inmunohistoquímicos, en el presente trabajo se han caracterizado las distintas poblaciones neuronales de la placa alar de la región del óbex, mediante el estudio de algunas de sus conexiones tanto aferentes como eferentes.

Aplicaciones del trazador en las raíces dorsales espinales.

En experimentos con aplicaciones de dextrano amina combinada con biotina (BDA) en raíces espinales cervicales y lumbares en *Pleurodeles waltl*, hemos observado que las aferencias primarias espinales, al entrar en la médula espinal, se dividen en un componente medial y otro lateral (tracto de Lissauer) cuyas fibras ascienden y descienden en el DF y DLF respectivamente. En niveles espinales ambos componentes inervan distintas regiones, si bien la atención del presente trabajo se centró, fundamentalmente, en el estudio de los componentes ascendentes que alcanzan la región del óbex.

Fibras del tracto de Lissauer correspondientes a la segunda raíz espinal, ascienden en el DLF hasta el polo rostral del complejo motor de los núcleos de los nervios craneales VII, IX y X, atravesando por lo tanto región del óbex. En su recorrido, algunas fibras alcanzan la banda celular externa donde se localiza el LCN, y en menor medida la banda celular interna, donde se sitúa el nVds. Igualmente observamos que las fibras del componente medial ascienden en el DF, organizadas somatotópicamente, confirmando datos previos aislados, basados en tinciones argénticas (Herrick, 1914; 1930; 1944), y de degeneración anterógrada (Nieuwenhuys y Cornelisz, 1971), y coincidiendo con el patrón de organización presente en anuros (ver capítulo 5 de la presente memoria). Las aferencias primarias procedentes de segmentos lumbares ascienden en la parte medial del DF, mientras que las aferencias braquiales lo hacen en posición más lateral. De la misma manera terminan en la región del óbex, principalmente en el DCN situado en la banda celular extrena, si bien un pequeño componente de fibras varicosas podría alcanzar al Nsol, nVds y LCN. Rostralmente al óbex, el componente medial de las aferencias braquiales asciende a través del rombencéfalo, hasta la capa granular del cerebelo.

Aplicaciones del trazador en el nervio trigémino

En experimentos con aplicaciones de BDA o de peroxidasa de rábano (HRP) en la raíz del nervio trigemino, se observó el recorrido descendente de algunas de sus aferencias primarias a través del Vds. En la placa alar de la región del óbex algunas fibras varicosas abandonan dicho tracto ventromedialmente, para dar terminales en las bandas celulares externa e interna, mayoritariamente en el nVds y en menor número en las zonas del DCN, Nsol y LCN.

Aplicaciones del trazador en el asta dorsal cervical

En nuestros experimentos con aplicaciones de BDA en niveles espinales cervicales, se marcaron fibras que discurren en el DF, DLF y Vds.

En el DF se observaron dos componentes: 1) El localizado en su porción medial, un tracto de fibras gruesas y agrupadas de manera muy compacta, que atraviesa la región del óbex sin dar ramas terminales, y termina en el lóbulo de la línea lateral. Dicho componente corresponde al tracto A de Kingsbury (1895), formado principalmente por aferencias primarias de la segunda raíz del complejo de los nervios IX-X (Roth y Wake, 1985), que inervan el sistema de la línea lateral (Kreht, 1930) y descienden hasta la médula espinal, donde se incorporan al DF (Kreht, 1930; Herrick, 1944; 1948; Roth y Wake, 1985). Herrick (1944; 1948) sugirió que el DCN podría recibir información del tracto A de Kingsbury, sin embargo, según nuestros resultados, esto parece poco probable, debido a que a nivel del óbex sus fibras, muy gruesas,

se disponen muy empaquetadas y no emiten colaterales. Además, en algunas especies de anuros, como *Xenopus laevis*, que retienen el sistema de la línea lateral en estadios adultos, sus aferencias no terminan en la región del DCN (Lowe y Russell, 1982; Altmany Dawes, 1983; Fritzsch y cols., 1984; Will y cols., 1985a). 2) El segundo componente ocupa una posición lateral en el DF y está formado por un sistema ascendente de fibras que terminan fundamentalmente en el DCN, si bien un menor número continúa rostralmente hasta la capa granular del cerebelo. Dicho sistema está constituído por aferencias primarias espinales, ya que las características de sus fibras coinciden con las que presentan las fibras marcadas en nuestros experimentos con aplicaciones en las raíces primarias espinales; aunque además podría incluir proyecciones no primarias, como las del sistema postsináptico de la columna dorsal, cuya presencia, aunque no se ha descrito hasta el momento en urodelos, ha sido demostrada en otros vertebrados, incluyendo los anuros (Rustioni, 1973; Angaut-Petit, 1975a,b; Uddenberg, 1968; Rustioni y Kaufman, 1977; Bennett y cols., 1984; Giesler y cols., 1974; Kamogawa y Bennett, 1986; Funke, 1988; ten Donkelaar y de Boer-van Huizen, 1991; Pritz y Stritzel, 1994; A. Muñoz y cols., 1995).

Las fibras ascendentes marcadas en el funículo dorsolateral, abandonan medialmente el tracto en niveles cervicales superiores y organizan un campo de terminales que alcanza el LCN. Dichas fibras corresponden a aferencias espinales primarias del tracto de Lissauer así como a proyecciones no primarias, que podrían incluir el tracto espinocervical, presente en anuros (ver capítulos 3 y 5 de la presente memoria).

Lemnisco medial

Existen muy pocos datos en la bibliografía sobre las conexiones eferentes del DCN en urodelos, y están basados únicamente en estudios realizados mediante tinciones argénticas. Herrick (1944; 1948) y Herrick y Bishop (1958) negaron la existencia del lemnisco medial en *Ambystoma tigrinum*, y compararon el DCN de urodelos con el núcleo cuneado externo, y no con los núcleos *gracilis* y *cuneatus* de mamíferos. Los citados autores decribieron proyecciones que se originan en la región del óbex y ascienden a través de los lemniscos espinal y general bulbar, a la formación reticular, cerebelo, techo mesencefálico y al tálamo dorsal; así como proyecciones ipsilaterales que alcanzan el cerebelo mezcladas con fibras de los tractos A y B de Kingsbury. Sin embargo, no existen trabajos experimentales, basados en técnicas de degeneración o de trazado neuronal, que confirmen las sugerencias de Herrick (1944, 1948). Únicamente en un estudio con aplicaciones de HRP en el torus semicircularis de *Salamandra salamandra* se describieron neuronas retógradamente marcadas en la región rombencefálica más caudal (Manteuffel y Naujoks-Manteuffel; 1990). Por el contrario en algunos trabajos se ha sugerido la ausencia en urodelos del lemnisco medial (Ebbesson y cols., 1972; Naujoks-Manteuffel y Manteuffel, 1986; Wicht y Himstdet, 1988).

Aplicaciones en el tálamo ventral y en el torus semicircularis.

En el presente estudio se han realizado aplicaciones de BDA en el tálamo ventral y en el torus semicircularis, con objeto de comprobar la posible existencia del lemnisco medial en *Pleurodeles waltl*, e identificar las neuronas que lo originan. En ambos casos se han observado en la placa alar de la región del óbex, neuronas retrógradamente marcadas,

mayoritariamente en el lado contralateral, en dos poblaciones neuronales diferenciables. La primera corresponde al DCN y se localiza en posición dorsomedial en la capa celular externa, a nivel del óbex y los primeros segmentos espinales. Dicha población está formada por neuronas de diferentes morfologías con dendritas orientadas hacia el funículo dorsal, a través del cual, presumiblemente, reciben información espinal ascendente. En los citados experimentos no se observó una división evidente entre componentes *gracilis* (medial) y *cuneatus* (lateral). La segunda población corresponde al LCN y se sitúa en posición ventrolateral en la capa celular externa, y se extiende desde el óbex hasta niveles ligeramente más caudales que el DCN. Sus neuronas tienen dendritas dirigidas lateralmente hacia el DLF, y en ocasiones algunas de sus neuronas se encuentran segregadas dentro de la propia sustancia blanca del DLF. En algunos casos se observaron células retrógradamente marcadas en posiciones intermedias entre ambas poblaciones, en el lugar donde se arborizan las aferencias trigeminales por lo que podrían corresponder al nVds. Los axones de la neuronas del DCN y LCN cruzan la línea media ventralmente al canal central, para inorporarse al funículo ventrolateral contralateral, y ascender a través del lemnisco medial hasta el torus semicircularis y el tálamo.

Además del tálamo y del torus semicircularis, el lemnisco medial de urodelos podría inervar otras regiones cerebrales como ocurre en otros vertebrados (ver capítulo 5 de la presente memoria). Algunos trabajos han descrito, por ejemplo, el procesamiento de información somatosensorial en el neuropilo profundo del techo óptico en diversas especies de urodelos (Grüsser-Cornehls y Himstedt, 1973; Gruberg y Solish, 1978; Gruberg y Harris, 1981; Harris, 1982, 1989; Stirling y Brandle, 1982; Roth y cols., 1990), que además de mediante las aferencias espinales (Naujoks Manteuffel y Manteuffel, 1988; Herrick, 1914, 1942, 1948; Nieuwenhuys y Cornelisz, 1971; Jakway y Riss, 1972; Gruberg, 1973; Gruberg y Solish, 1978; Finkenstadt y cols., 1983; Rettig, 1988, 1989; A. Muñoz y cols. capítulo 3 de la presente memoria) podrían recibirla a través de las aferencias somatosensoriales procedentes de la región del óbex, como en anuros (capítulo 5 de la presente memoria). Cabe resaltar que Finkenstädt y cols. (1983), en experimentos con aplicaciones de HRP en el techo óptico de *Salamandra salamandra*, observaron neuronas retrógradamente marcadas en los segmentos espinales más rostrales, en una localización similar a la descrita en este estudio para el DCN.

Así pues en el presente trabajo se ha demostrado la existencia del lemnisco medial en urodelos, mediante el estudio de sus proyecciones al tálamo ventral y al torus semicircularis. Sin embargo, resulta necesaria la aplicación de trazadores anterógrados en la región del DCN y del LCN, para conocer la anatomía detallada del lemnisco medial en urodelos así como la totalidad de los centros a los que pueda proyectar.

CAPÍTULO 7

1- Se ha demostrado la **viabilidad de preparaciones *in vitro*** en las que el sistema nervioso central de anfibios, completo y aislado del cuerpo del animal, puede mantenerse vivo durante varios días en unas condiciones que permiten la realización de experimentos anatómicos y electrofisiológicos, cuyos **resultados son comparables** a los obtenidos con preparaciones *in vivo*.

2- Mediante trazado neuronal así como técnicas histoquímicas e inmunohistoquímicas, se han caracterizado en anuros y urodelos, los **núcleos de la columna dorsal, cervical lateral, descendente del nervio trigémino y del tracto solitario.**

3- En anuros y en urodelos las proyecciones somatosensoriales espinales ascendentes se organizan, al igual que en vertebrados amniotas, en **tres componentes: funículo dorsal**, funículo **dorsolateral** y **cuadrante ventral** (funículos dorsal y dorsolateral).

4- El **funículo dorsal** de anuros y urodelos posee **fibras primarias** procedentes de las neuronas ganglionares espinales, en anuros incluye además fibras **no primarias** del **sistema postsináptico de la columna dorsal.** Todos los componentes presentan una **somatotopía mediolateral** y terminan mayoritariamente en el **núcleo de la columna dorsal.**

5- **El funículo dorsolateral** de los anfibios estudiados está formado por fibras **primarias** del **tracto de Lissauer** y fibras ascendentes **no primarias**. En las últimas se incluyen el **tracto espinocervical,** que termina en el **núcleo cervical lateral** así como otras fibras que rostralmente alcanzan el cerebelo y distintos centros del tronco cerebral como la **formación reticular lateral** y el **área parabraquial.**

6- Tanto en anuros como en urodelos se ha demostrado la existencia, en el **cuadrante ventral,** de **proyecciones espinotalámicas** directas que alcanzan diversos núcleos del tálamo dorsal y ventral. Así como proyecciones que terminan en distintos núcleos del **torus semicircularis** que, junto con las del lemnisco medial, constituyen el sustrato anatómico por el que la información somatosensorial alcanza dicho centro, en el que se ha descrito un mapa somatotópico de representación de la superficie corporal.

7- Los **núcleos de la columna dorsal** y **cervical lateral** proyectan, en anuros y urodelos, a través del **lemnisco medial,** al **torus semicircularis** y al **tálamo.**

8- En *Xenopus laevis* el **desarrollo** de las aferencias al núcleo de la columna dorsal precede al de sus eferencias al mesencéfalo y al tálamo, por lo que se puede considerar que existe un **patrón de determinación periférico-central** en el sistema columna dorsal-lemnisco medial.

9- Los datos hodológicos e inmunohistoquímicos obtenidos demuestran la existencia, tanto en anuros como en urodelos, de los sistemas **columna dorsal-lemnisco medial** y **espino-cervico-talámico, comparables** a los descritos en vertebrados amniotas.